	Type	L #	Hits	Search Text	DBs	Time Stamp	Comments	Error Definition	Errors
1	BRS	L1	4664	her2 or her-2 or cerbb2 or c-erbb2 or c-erbb-2 or cerbb-2 or p185 or neu or herceptin or trastuzumab	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2002/01/22 17:25			0
2	BRS	L2	7866	(PROSTATE OR PROSTATIC) near3 (CANCER OR CARCINOMA OR TUMOR OR TUMOUR OR NEOPLAS$3 OR MALIGNAN$4)	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2002/01/22 17:26			0
3	BRS	L3	74	1 same 2	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2002/01/22 17:26			0

	Type	L #	Hits	Search Text	DBs	Time Stamp	Comments	Error Definition	Errors
4	BRS	L4	23	1 with 2	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2002/01/22 17:26			0

(FILE 'HOME' ENTERED AT 17:11:36 ON 22 JAN 2002)

FILE 'MEDLINE, EMBASE, BIOSIS, CAPLUS, CANCERLIT, SCISEARCH' ENTERED AT 17:12:00 ON 22 JAN 2002

L1 33844 S HER2 OR HER-2 OR CERBB2 OR C-ERBB2 OR C-ERBB-2 OR CERBB-2
OR
L2 186734 S (PROSTATE OR PROSTATIC) (3A) (CANCER OR CARCINOMA OR TUMOR
OR
L3 511 S L1 (30A) L2
L4 4220 S ((HER2 OR HER-2 OR CERBB2 OR C-ERBB2 OR C-ERBB-2 OR CERBB-2
O
L5 71 S L4 (30A) L2
L6 30 DUP REM L5 (41 DUPLICATES REMOVED)

STIC-ILL

R1261.A4313

From: Hunt, Jennifer
Sent: Tuesday, January 22, 2002 5:54 PM
To: STIC-ILL
Subject: References for 09/602,800

Please send me the following references ASAP:

Hum. Gene Ther. (1997), 8(2), 157-170

CLINICAL CANCER RESEARCH, (1999 Aug) 5 (8) 2171-7

CANCER RESEARCH, (1999 Oct 1) 59 (19) 4761-4

SEMINARS IN ONCOLOGY, (2000 Dec) 27 (6 Suppl 11) 53-63; discussion 92-100

SEMINARS IN ONCOLOGY, (2001 Aug) 28 (4 Suppl 15) 3-7

SEMINARS IN ONCOLOGY, (2001 Aug) 28 (4 Suppl 15) 71-6

Thanks.

Jennifer Hunt
Patent Examiner, Art Unit 1642
CM1-8D06 (mailbox 8E12)
(703)308-7548

HER-2/*neu* as a Therapeutic Target in Non–Small Cell Lung Cancer, Prostate Cancer, and Ovarian Cancer

David B. Agus, Paul A. Bunn, Jr, Wilbur Franklin, Marileila Garcia, and Robert F. Ozols

HER-2/*neu* is overexpressed in most epithelial malignancies. Lung cancer, prostate cancer, and ovarian cancer are common epithelial tumors in which clinical trials are currently in progress to explore the potential therapeutic role for monoclonal antibodies to HER-2/*neu* (trastuzumab [Herceptin; Genentech, Inc, South San Francisco, CA]). In preclinical studies with tumor cell lines, trastuzumab was found to have additive and synergistic effects with some chemotherapeutic agents. Clinical trials investigating combination chemotherapy with trastuzumab and a variety of chemotherapeutic agents are already in progress in lung cancer.
Semin Oncol 27 (suppl 11):53-63. *Copyright © 2000 by W.B. Saunders Company.*

HER-2/*neu* is overexpressed in most epithelial malignancies, especially adenocarcinomas.[1] Lung cancer, ovarian cancer, and prostate cancer are no exceptions. In this report we will examine the potential therapeutic role for monoclonal antibodies to HER-2/*neu* in these common malignancies, either as a single agent or in combination with chemotherapy.

EXPRESSION OF HER-2/*neu* IN LUNG CANCER

HER-2/*neu* is rarely overexpressed in small cell carcinomas, is sometimes overexpressed in large cell and squamous carcinomas, and is frequently overexpressed in adenocarcinomas.[2,3] In cell lines of adenocarcinomas of the lung that overexpress HER-2/*neu*, monoclonal antibodies to HER-2/*neu*, such as trastuzumab (Herceptin; Genentech, Inc, South San Francisco, CA), receptor HER-2/*neu*–specific tyrosine kinase inhibitors, and immunotoxins inhibit growth both in vitro and in vivo.[4-6] The monoclonal antibody trastuzumab produces synergistic growth inhibition with a number of chemotherapeutic agents such as the taxanes and gemcitabine. These facts have led to a number of clinical trials of trastuzumab alone and in combination with chemotherapeutic agents in untreated patients with advanced non-small cell lung cancer (NSCLC) and in previously treated patients.

Table 1 summarizes published studies reporting on the expression of HER-2/*neu* in lung cancer cell lines.[7-11] It is obvious that overexpression is highest in non-small cell histologies including adenocarcinoma, squamous, and large-cell tumors, but is rare in small cell lung cancer (SCLC) cell lines. The frequency of overexpression in adenocarcinoma of the lung is similar to that seen in breast cancer cell lines. The experience at the University of Colorado Cancer Center in studies of HER-2/*neu* expression in lung cancer cell lines is summarized in Table 2. As in studies from the literature, the frequency of overexpression is similar in adenocarcinoma lines of breast or lung origin. The degree of overexpression, as expressed by the mean fluorescence intensity determined by FACS analysis, is less in the lung cancer cell lines. Large cell and squamous cell lung cancer cell lines overexpressed HER-2/*neu*, but with a lower percentage of positive cells and a lower fluorescence intensity. No overexpression of HER-2/*neu* was observed in SCLC cell lines.

Studies of HER-2/*neu* expression in lung cancer samples are summarized by histology in Table 3.[3,7-9,12-24] Figure 1 shows an example of a human adenocarcinoma of the lung stained with an antibody to HER-2/*neu* compared with an isotype-matched control. As in the cell line studies, overexpression is limited to patients with adenocarcinoma, and approximately 30% of tumors overexpress HER-2/

From the Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY; the University of Colorado Cancer Center, Denver, CO; the Departments of Anatomic Pathology and Medical Oncology, the University of Colorado Health Sciences Center, Denver, CO; and the Department of Medical Science, Fox Chase Cancer Center, Philadelphia, PA.

Supported in part by National Cancer Institute grant nos. NCIP50CA58187 and NCIP30CA46934, CaPCURE, and the Eleanor and Paul Stephens Foundation.

Dr Agus has served on the speaker's bureau for Genentech, Inc. Dr Bunn has received research grant support from Bristol-Myers Squibb, Cell Pathways, and Eli Lilly, he has served as a paid consultant to Eli Lilly, Seragen, Smith-Kline Beecham, US Bioscience, Rhone-Poulenc Rorer, and Upjohn Pharmaceuticals.

Address reprint requests to David B. Agus, MD, Cedars-Sinai Medical Center, Prostate Cancer Center, 8631 W Third St, Suite 1001E, Los Angeles, CA 90048.

Copyright © 2000 by W.B. Saunders Company
0093-7754/00/2706-1110$35.00/0
doi:10.1053/sonc.2000.19898

Table 1. HER-2/*neu* Expression in Lung Cancer Cell Lines

Cell Type	Reference	Mean No. Tested	Mean No. Positive	Analysis
Adenocarcinoma	7-11	10	6	WBA, IP, IA
Squamous	7-11	3	2	NBA, IP, IA, WBA
Large	7-9,11	5	2	NBA, IP, IA, WBA
SCLC	9	3	0	IP

Abbreviations: NBA, Northern blot analysis; WBA, Western blot analysis; IP, immunoprecipitation; IA, immunoassay.

neu. The degree of overexpression may be less than in breast cancer but is similar to that in prostate and ovarian cancer. Small cell lung cancers rarely, if ever, overexpress HER-2/*neu*.

PROGNOSTIC IMPLICATIONS

It is well established that overexpression of HER-2/*neu* in breast cancer is associated with a worse prognosis.[25] Although there are fewer studies in lung cancer, multiple published studies suggest that HER-2/*neu* overexpression also is associated with a worse prognosis in lung cancer patients.[21-24]

GROWTH INHIBITION BY TRASTUZUMAB

Several groups, including our own, have studied in vitro growth inhibition of lung cancer cell lines by antibodies/immunotoxins to HER-2/*neu*.[4,5] Figure 2 shows the growth of human breast and lung cancer cell lines exposed to varying concentrations of trastuzumab. Clearly, the degree of growth inhibition is quite similar and dependent on the duration of exposure and concentration. The degree of growth inhibition of the NSCLC cell lines was not as great as with high-expressing breast cancer cell lines such as SKBR3, especially with long duration of exposure, but lung cancer lines were growth-inhibited to a similar extent as most breast cancer lines.

We investigated the interactions of trastuzumab and various chemotherapeutic agents on the growth of lung cancer cell lines in vitro. An isobologram method developed by Chou and Talalay[26] was used to analyze the results. We found that trastuzumab produces additive or synergistic effects on adenocarcinoma cell lines that overexpress HER-2/*neu*, whereas there was no positive interaction with nonexpressing small cell lines. An example is shown in Fig 3 where gemcitabine shows synergistic interactions with trastuzumab in breast and lung cancer adenocarcinoma cell lines but not in small cell carcinoma cell lines.

IN VIVO STUDIES

Several studies have evaluated the growth inhibition produced by other inhibitors of HER-2/*neu*, such as a recombinant toxin that was used in human lung cancer cell lines grown in athymic nude mice.[5] These studies invariably showed in vivo growth inhibition that was dose related. An example is shown in Fig 4 where OLX-209 inhibited the growth of four human lung cancers.

Table 2. FACS Analysis of HER-2/*neu* Expression in Lung Cancer Cell Lines

Cell Line	% HER-2/*neu*+	MFI
Adenocarcinoma Lines		
A549	72%	3.7
H322	98%	9.8
H324	98%	8.0
Squamous Carcinoma Lines		
H157	62%	2.8
NE18	93%	3.9
Breast Carcinoma Lines		
SKBR3	100%	29.0
ZR75	100%	13.0
T47-DV	98%	11.0
Small Cell Carcinoma Lines		
H345	0%	–
H209	0%	–
H187	0%	–
Large Cell Carcinoma Lines		
H1334	74%	3.0
H460	57%	2.4

Abbreviation: MFI, mean fluorescence intensity.

Table 3. HER-2/*neu* Expression in Lung Cancer

Cell Type	Reference	Mean No. Tested	Mean % Positive	Analysis
Adenocarcinoma	3,7,8,12-18	45	25%	SBA, IP, IHC
Squamous	3,7,8,12-14,16,17,19	30	32%	SBA, IP, IHC, NBA
Large	3,7,8,12,13	9	31%	SBA, IP, IHC, NBA
NSCLC	9,20-24	175	28%	SBA, IP, PCR
SCLC	6,20	6	0%	SBA, IHC

Abbreviations: SBA, Southern blot analysis; IP, immunoprecipitation; IHC, immunohistochemistry; NBA, Northern blot analysis; PCR, polymerase chain reaction.

MECHANISM OF HER-2/*neu* OVEREXPRESSION

The mechanism of overexpression of HER-2/*neu* in human lung cancer cell lines was examined using fluorescence in situ hybridization (FISH), with probes for the centromere of chromosome 17 and for the region on chromosome 17 where HER-2/*neu* resides. Data for a representative NSCLC cell line NCI-H322 (an adenocarcinoma cell line) are shown in Fig 5. NCI-H322 has a near-tetraploid DNA content, with a chromosome range of 110 to 132 chromosomes/cell. The overexpression of HER-2/*neu* was closely related to the increased copy number, as there were 5.7 chromosome 17 centromeres/cell and 6.5 copies of HER-2/*neu*/cell for a gene-chromosome ratio of 1.1. By FACS analysis, 98% of the H322 cells expressed HER-2/*neu*. In the SCLC cell lines the HER-2/*neu* gene was generally expressed, often with increased copy number, but the cell surface protein was not expressed, indicating dysregulation in transcription, translation, or processing.

CLINICAL TRIALS IN NON-SMALL CELL LUNG CANCER

Because many human lung cancers overexpress HER-2/*neu*, overexpressing lines can be growth inhibited in vitro and in vivo by trastuzumab, and HER-2/*neu* overexpression is associated with a poor prognosis, it is logical to study trastuzumab in patients with NSCLC. A number of groups are now instituting clinical trials. In essentially all of these trials, accrual is limited to patients whose tumors have 2+ to 3+ expression of HER-2/*neu* as tested by a central laboratory using immunohistochemistry. Some studies are proposed in untreated patients. A list of these trials is summarized in Table 4. In all of these trials, trastuzumab is given at a loading dose of 4 mg/kg intravenously on the first week, then weekly at 2 mg/kg. As shown, the trials propose to use trastuzumab alone and in combination with a variety of other chemotherapeutic agents.

Other trials are proposed in patients for second-line therapy as shown in Table 5. Once again, trials of trastuzumab alone and combined with other agents are planned. The response rate to standard chemotherapeutic agents is generally less than 10% in these patients, and a response rate of 20% to a combination would appear promising. The University of Colorado Cancer Center plans to study gemcitabine in combination with trastuzumab because of the synergy noted in vitro and because there are no clinical trials of this combination.

OVARIAN CANCER

Ovarian cancer was one of the first malignancies in which amplification/expression of HER-2/*neu* was felt to have prognostic significance. In 1989, Slamon et al[27] showed a decrease in survival in ovarian cancer patients whose tumors had gene amplification or increased expression of HER-2/*neu*. Since that initial report there have been at least seven more publications exploring the relationship between HER-2/*neu* expression and prognosis in patients with ovarian cancer; these reports are summarized in Table 6.[27-34]The percentage of epithelial ovarian cancer with increased HER-2/*neu* expression ranged between 20% and 40%, with a median of 26%. However, there was no agreement as to the prognostic significance of expression, with three studies reporting an adverse effect on prognosis.

The reasons for the disparate results remain to be determined and may reflect different reagents

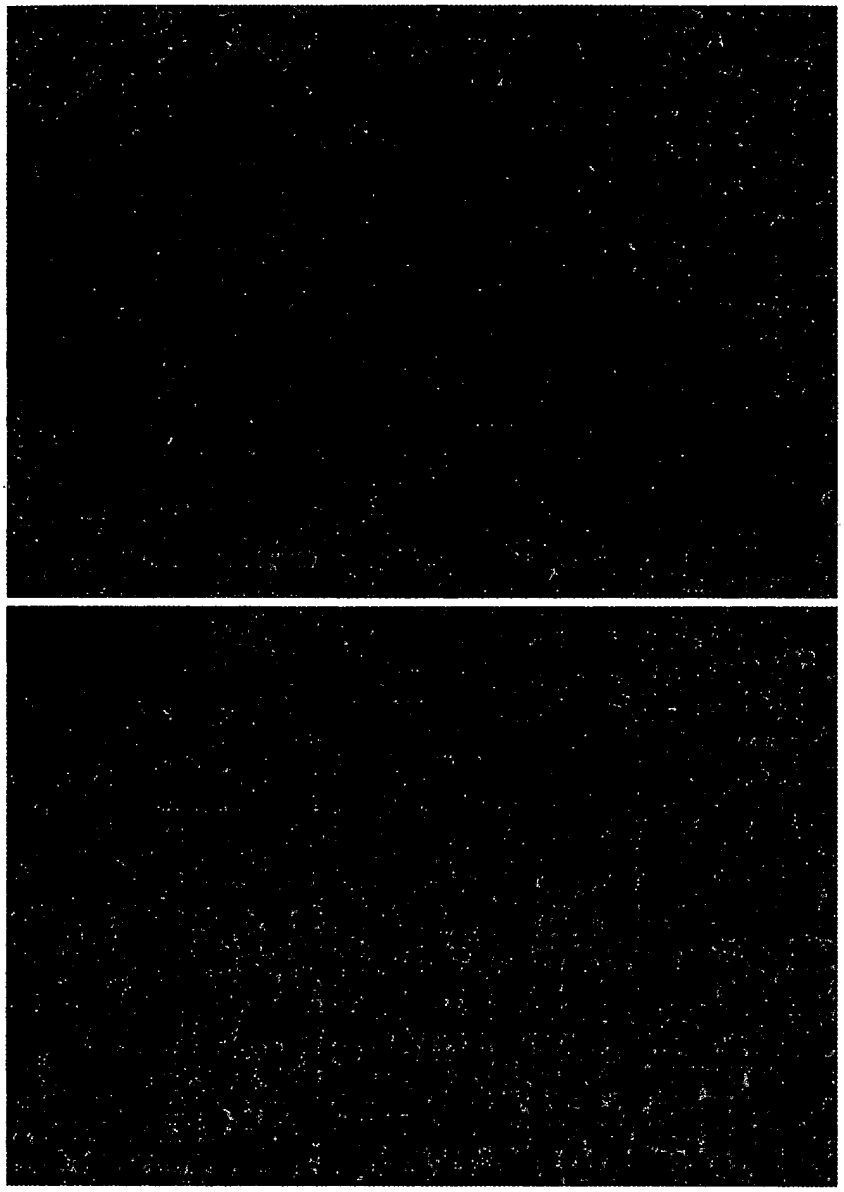


Fig 1. Immunohistochemical assay for HER-2/*neu*. A human lung adenocarcinoma was stained with an anti–HER-2/*neu*–specific monoclonal antibody or the isotype-matched control antibody. Lung adenocarcinoma showing strong anti–HER-2/*neu* staining (top). The same lung adenocarcinoma showing no reactivity with the isotype-matched control (bottom).

used to determine immunoreactivity. The Gynecologic Oncology Group is performing a large study on the prognostic effect of HER-2/*neu* expression in which tumors from all patients are similarly evaluated for immunoreactivity.

The predictive value of expression of HER-2/*neu* in ovarian cancer also remains to be determined. Preclinical data have shown there is a synergistic effect on cell kill between antibodies directed against HER-2/*neu* and platinum compounds.[35,36] The mechanism resulting in the synergy remains to be established, although it appears that antibodies against HER-2/*neu* may interfere with the tumor cells' capacity to repair DNA damage associated with the formation of platinum adducts. Currently, the best available chemotherapy regimen for ovarian cancer consists of the combination of the platinum compound and paclitaxel.[37] It is tempting to speculate that, analogous to the observed clinical benefit in breast cancer, combination treatment with trastuzumab, a platinum compound, and paclitaxel may produce even more clinical benefit in ovarian cancer patients whose tumors overexpress HER-2/*neu*. However, there

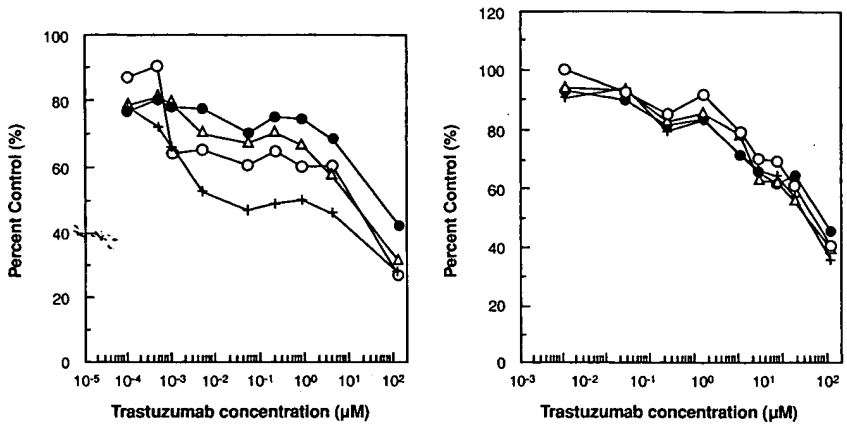


Fig 2. Trastuzumab inhibits the growth of breast and lung cancer cell lines. Breast cancer cell line SKBR3 (left). Non–small cell lung cancer cell line A549 (right). Each line was exposed to varying concentrations of trastuzumab for varying lengths of time. Growth inhibition was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide assay. Solid circle, 48 hours of drug exposure; triangle, 72 hours of drug exposure; open circle, 96 hours of drug exposure; +, 120 hours of drug exposure.

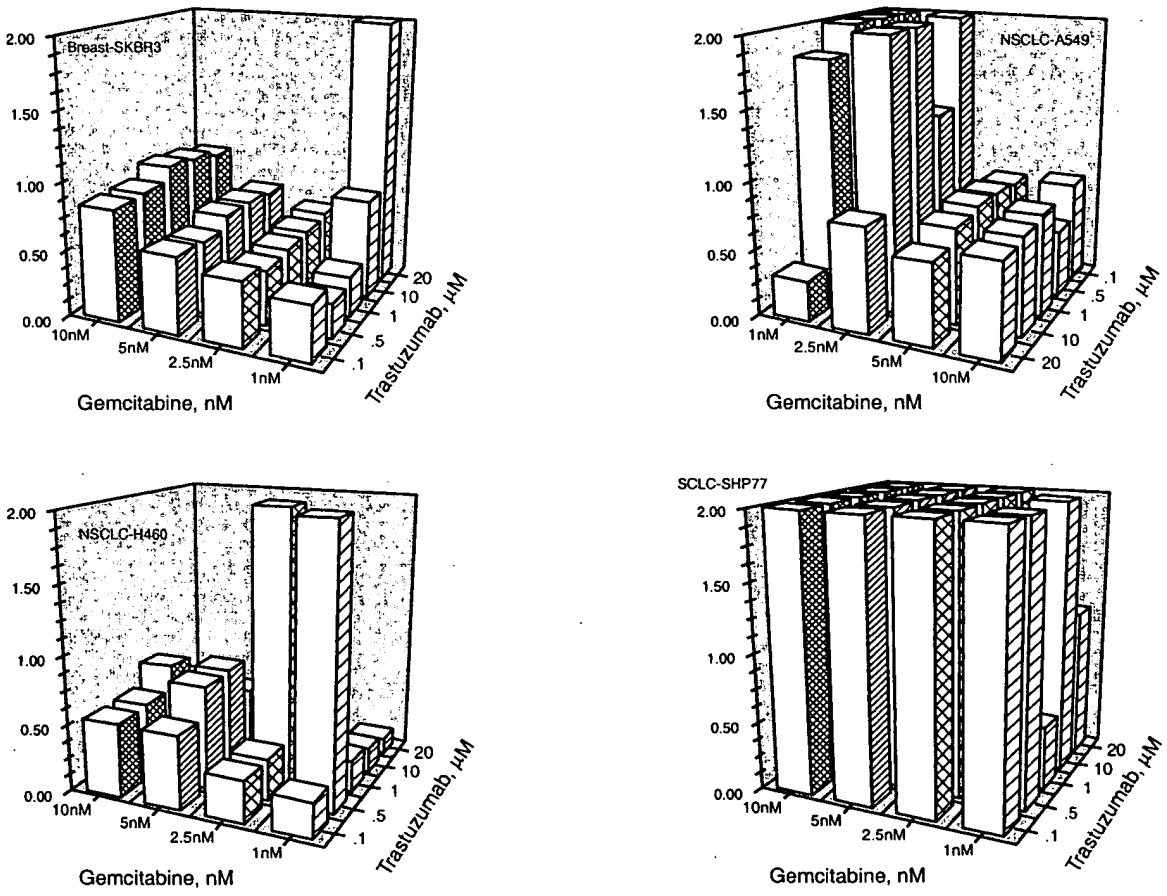


Fig 3. Synergistic effects of trastuzumab plus gemcitabine in breast and NSCLC lines. Antagonistic effects of trastuzumab plus gemcitabine in SCLC. The two drugs were added simultaneously to each cell line. Following a 5-day incubation, MTT was added and the plates were harvested 4 hours later. A combination-index (CI) in which CI = 1 indicates an additive effect, CI < 1 indicates a synergism between the two drugs, and CI > 1 indicates antagonism between the two drugs.

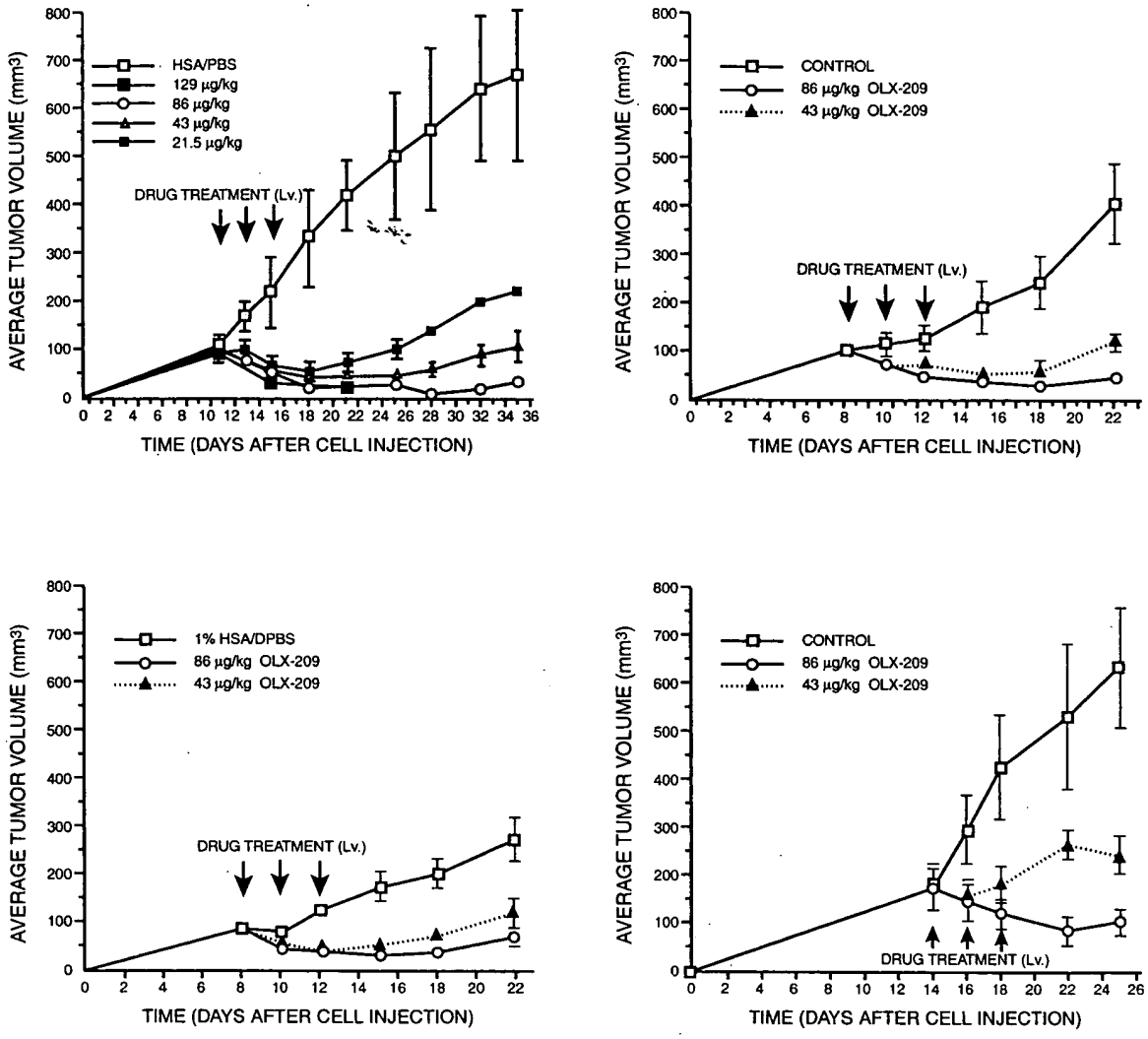


Fig 4. Therapy for tumors formed in mice by human lung adenocarcinomas cell lines Calu-3 (top left), NCI-H1446 (top right), A549 (bottom left), and 201T (bottom right). Tumors were established by subcutaneous injection of 5 x 10^6 cells in the backs of athymic nude mice. Therapy was initiated 8 to 14 days following injection of cells. Treatment with OLX-209 (at least five mice/group) was given intravenously three times, once every 2 days. Tail vein injection was given with 1% human serum albumin/PBS. Measurements were conducted externally with calipers. Tumor growth is reported as an average relative tumor volume. (Reprinted with permission.[5])

currently are no published clinical data to support the use of such a combination.

GYNECOLOGIC ONCOLOGY GROUP TRIALS OF TRASTUZUMAB

The Gynecologic Oncology Group has initiated an evaluation of trastuzumab in recurrent or refractory ovarian cancer for patients with a primary peritoneal carcinoma (Gynecologic Oncology Group Protocol 160).[38] This is a phase II open-label study to determine the safety and efficacy of trastuzumab in metastatic ovarian or primary peritoneal carcinoma patients who have persistent or recurrent disease following initial treatment with platinum-based cytotoxic chemotherapy and whose tumors overexpress HER-2/*neu*.

Patients also must have bidimensionally measurable disease. Trastuzumab is administered as a 4 mg/kg intravenous loading dose on day 1 and weekly thereafter at a dose of 2 mg/kg until disease progression. Patients who have a favorable response after the initial 8 weeks (either complete

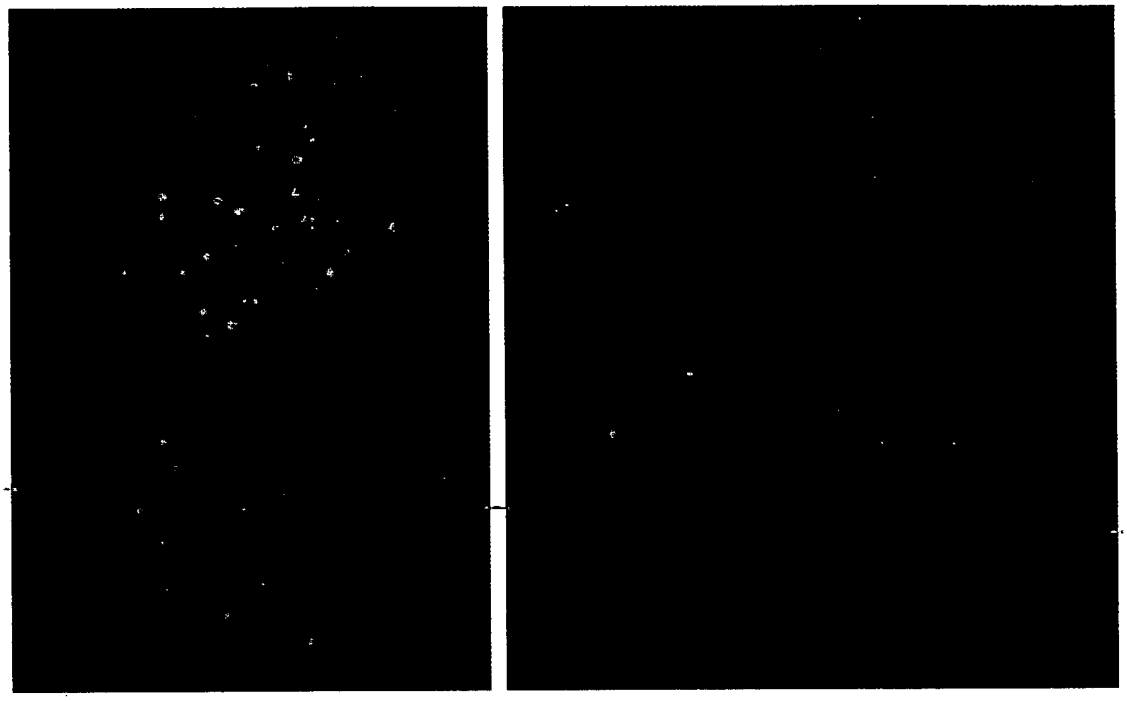


Fig 5. Dual-color interphase (left) and metaphase (right) FISH analysis was performed on the NSCLC cell line H322. Red staining is from the HER-2/*neu* probe, and green staining is from the centromere 17 probe. There were 5.7 chromosome 17 centromeres/cell and 6.5 copies of the HER-2/*neu* gene/cell.

response, partial response, or stable disease) and then develop progressive disease will be eligible to be retreated with a dose escalation to 4 mg/kg weekly. All patients will be followed for survival.

Statistically, this protocol has a two-stage sampling design with appropriate early stopping rules. In the first stage of the protocol, 25 patients will be accrued. If the observed number of responses is three or less, it is unlikely that the protocol will continue. However, if the observed number of responses is greater than three, and if medical judgment supports the decision, a second state of accrual will begin in which 15 additional patients will be accrued. The antibody will be considered active if the total observed number of responses is seven or more.

Subsequently, it is planned that additional studies will be performed with trastuzumab together

Table 4. Trastuzumab Trials in Advanced, Untreated Non-Small Cell Lung Cancer

Institution (Principal Investigator)	Chemotherapy	Trial Size
Univ. of Pittsburgh (Belani)	Paclitaxel + carboplatin weekly	40
M.D. Anderson (Herbst)	Gemcitabine + cisplatin every 3 wks	100
MSKCC (Miller)	Docetaxel every 3 wks	100
Univ. of California, Davis (Gandara)	Docetaxel weekly	40
SWOG (Gandara)	Trastuzumab → paclitaxel + carboplatin + trastuzumab	100
ECOG* (Johnson)	Paclitaxel + carboplatin ± trastuzumab	100

Abbreviations: MSKCC, Memorial Sloan-Kettering Cancer Center; SWOG, Southwest Oncology Group; ECOG, Eastern Cooperative Oncology Group.

*Randomized trial.

Table 5. Trastuzumab Trials in Advanced, Second-Line Non-Small Cell Lung Cancer

Institution (Principal Investigator)	Chemotherapy	Trial Size
CALGB (Kern)	None	40
Presbyterian/St. Luke's of Chicago (Bonomi)	None	80
Univ. of Colorado (Kelly)	Gemcitabine weekly	40
SWOG (Gandara)	Docetaxel every 3 wks	50

Abbreviations: CALGB, Cancer and Leukemia Group B; SWOG, Southwest Oncology Group.

with chemotherapy to determine if synergy can be shown either with platinum compounds, paclitaxel, or the combination in patients with recurrent ovarian cancer. The major strength of this study will be that it will prospectively determine the percentage of patients with epithelial ovarian cancer whose tumors overexpress HER-2/*neu*. In addition, it will establish a large phase II trial to determine whether the antibody alone has any activity in recurrent platinum-resistant ovarian cancer patients.

HER2/*neu* EXPRESSION IN PROSTATE CANCER

There are disparate data with regard to HER-2/*neu* expression in prostate cancer.[39-42] Ross et al[40] showed that amplification of HER-2/*neu* correlated with Gleason score, with the mean Gleason score of HER-2/*neu* unamplified tumors being 5.7 and that of HER-2/*neu* amplified tumors being 7.5. In this study, 62 cases were evaluated by immunohistochemistry (IHC) and FISH: 18 cases (29%) were overexpressed by IHC, and 27 cases (44%) were amplified by FISH.[40] A trend for similar HER-2/*neu* status in each prostate cancer by the two methods did not reach statistical significance. Sadasivan et al[41] noted HER-2/*neu* overexpression by IHC in 9 of 25 prostatic adenocarcinoma samples. In this study, overexpression was correlated with higher histologic grade, higher stage of disease, and a high S-phase and aneuploidy by flow cytometric analyses.[41] Kuhn et al[43] showed overexpression of HER-2/*neu* via IHC on 34% (18 of 53 cases) of prostatic adenocarcinomas. Overexpression of HER-2/*neu* did not appear to be a prognostic marker for prostate cancer outcome in this study. Zhau et al[44] showed 12 of 15 prostate

Table 6. HER-2/*neu* Expression in Ovarian Cancer: Prognostic Significance

Study	Patient Characteristics	Positive for HER-2/*neu* Expression	Prognostic Significance
Slamon et al[27]	120 primary ovarian cancer	31/120 (26%) with amplification/expression	Statistically significant decrease in survival with amplification/expression.
Berchuck et al[28]	73 advanced stage (III-IV)	23/73 (32%) with 3+ staining	Statistically significant decrease in survival for positive staining: median survival 15.7 mo *v* 32.8 mo.
Scambia et al[29]	94 advanced stage (III-IV)	33/94 (35%) with positive staining	No correlation with survival.
Rubin et al[30]	105 stage III-IV	25/105 (24%) with 3+ staining	No survival differences.
Felip et al[31]	106 stage I-IV	23/106 (22%) with overexpression	Increased overexpression in advanced stage *v* early stage and in patients not responding to surgery. Worse survival in overexpressors.
Fajac et al[32]	65 stage I-IV	Overexpression in 23/52 (44%) adenocarcinomas	No difference in survival for overexpressors in univariate and multivariate analysis.
van der Zee et al[33]	89 primary tumors	18/89 (20%) immunoreactive	No association with progression-free and overall survival.
van Dam et al[34]	80 primary ovarian cancer	19/80 (24%) increased expression	Increased expression in recurrent disease.

cancer specimens overexpressed HER-2/*neu* by IHC, which was not detectable in normal prostate tissues. Visakorpi et al[45] showed no HER-2/*neu* reactivity by IHC in 147 paraffin-embedded malignant prostatic tissues.[45] Thus, there are disparate results in the literature with regard to HER-2/*neu* expression in prostate cancer. This is a reflection of the wide range of anti–HER-2/*neu* antibodies used, a wide range of tissue preparations, and a wide range of tumor grades analyzed in these studies. In addition, the grading system used in these studies was not uniform. Many studies use grading systems validated for breast cancer samples, but these may not be applicable to prostate cancer. There are several methods of testing for HER-2/*neu* in prostate cancer, including IHC and FISH analyses. A study by Ross et al[42] showed HER-2/*neu* gene amplification by FISH to be more sensitive than IHC in detecting HER-2/*neu* gene abnormalities. This study was performed on 113 formalin-fixed prostate cancer specimens.[42] There are currently ongoing studies evaluating HER-2/*neu* overexpression using the Dako IHC kit (Dako Corp, Carpinteria, CA), which is approved by the Food and Drug Administration for breast cancer specimens. In addition, studies are ongoing comparing the Dako IHC kit with other HER-2–directed monoclonal antibodies, as well as FISH analysis. Another point to consider in the published studies is that most were performed on prostatectomy tissues or transurethral prostatectomy samples and very few were performed on metastatic, androgen-independent disease. Also, heterogeneity in HER-2/*neu* expression has been noted with prostate xenograft tumors as well as primary tumor specimens.[46] The heterogeneity of cell surface staining appears to be distinct from breast cancer HER-2/*neu* staining.

Clinical trials are currently ongoing with trastuzumab in prostate cancer targeted to androgen-dependent as well as androgen-independent patients. A common problem in these clinical trials is that the HER-2/*neu* status of the patient is commonly determined by looking at primary prostatectomy or transurethral prostatectomy samples rather than the metastatic disease. Thus, when a patient with androgen-independent disease is treated with trastuzumab and is termed HER-2 positive or negative, this is generally reflective of the primary disease and may or may not reflect current disease status. The lack of biopsy specimens from androgen-independent disease is important to consider in designing clinical trials and selecting appropriate patients.

CLINICAL UTILITY OF TRASTUZUMAB IN PROSTATE CANCER

Because HER-2/*neu* may be overexpressed in prostate cancer, we examined the effect of trastuzumab monotherapy in androgen-dependent (CWR22 and LNCaP) and androgen-independent (CWR22 derivatives) prostate cancer xenograft models[47-49] and the combination of trastuzumab with paclitaxel.[46] We found that trastuzumab alone has antitumor activity in the androgen-dependent human prostate cancer xenograft models studied, and has at least an additive effect with paclitaxel in terms of growth inhibition. Trastuzumab alone had no demonstrable antitumor effect in the androgen-independent models, but clearly enhanced the activity of paclitaxel.

Recently it has been shown that overexpression of HER-2/*neu* can allow androgen-independent growth in LNCaP xenograft models.[50] All of the androgen-independent xenografts used in the described studies expressed androgen receptor, as assayed by IHC, and overexpressed HER-2/*neu* on the cell surface. The expression of functional androgen receptor, coupled with the overexpression of HER-2/*neu* in the parental tumor cell line CWR22, suggests that HER-2/*neu* is not expressed because of androgen insensitivity in these tumors. The lack of response to trastuzumab in the androgen-independent tumors suggests that the androgen signaling pathway in the androgen-dependent models may be permissive with regard to trastuzumab response. The basis for the ability of trastuzumab to enhance the paclitaxel antitumor effect in androgen-independent prostate cancer is uncertain. It is possible that trastuzumab initiates or impairs cell signaling pathways that allow for increased paclitaxel cell kill. Other mechanisms may include immunologic effects or an effect of trastuzumab on the tumor concentration of paclitaxel. Growth inhibition in androgen-dependent tumors by trastuzumab was accompanied by an increased prostate-specific antigen (PSA) index when compared with pretreatment values. In contrast, the control-treated animals showed a decrease in the PSA index with time. HER-2/*neu*

overexpression in prostate cancer cells may cause androgen-independent growth by activation of the androgen receptor signaling pathway in a ligand-independent fashion.[50] In the CWR22 model systems, trastuzumab binding to HER-2/*neu* led to increased cellular secretion of PSA in an androgen-independent fashion. This result further supports the notion of crosstalk between the HER-2/*neu* and androgen receptor signaling pathways. The lack of correspondence between the PSA index and tumor response will complicate clinical trial design, because PSA is routinely used as a surrogate for tumor response. Clinical trials are currently underway examining the role of trastuzumab in human prostate cancer using endpoints other than PSA. Ongoing clinical trials will help to determine the HER-2/*neu* expression patterns in prostate cancer. In addition, the clinical utility of trastuzumab in prostate cancer, as monotherapy and in combination with chemotherapy, is being explored in clinical trials with patients who have androgen-dependent and androgen-independent prostate cancer.

REFERENCES

1. Salgia R, Sarkin AT: Molecular abnormalities in lung cancer. J Clin Oncol 16:1207- 1217, 1998
2. Kiefer PE, Bepler G, Kubasch M, et al: Amplification and expression of protooncogenes in human small cell lung cancer cell lines. Cancer Res 47:6236-6242, 1987
3. Lorenz J, Friedberg T, Paulus R, et al: Oncogene overexpression in non–small cell lung tissue: Prevalence and clinicopathological significance. Clin Invest Med 72:156-163, 1994
4. Kern JA, Torney L, Weiner D, et al: Inhibition of human lung cancer cell line growth by an anti-p185HER2 antibody. Am J Respir Cell Mol Biol 9:448-454, 1993
5. Kasprzyk PG, Sullivan TL, Hunt JD, et al: Activity of anti–erbB-2 recombinant toxin OLX-209 on lung cancer cell lines in the absence of erbB-2 gene amplification. Clin Cancer Res 2:75-80, 1996
6. Zhang L, Hung MC: Sensitization of HER-2/*neu*-overexpressing non–small cell lung cancer cells to chemotherapeutic drugs by tyrosine kinase inhibitor emodin. Oncogene 12:571-576, 1996
7. Weiner DB, Nordberg J, Robinson R, et al: Expression of the *neu* gene-encoded protein (P185neu) in human non–small cell carcinomas of the lung. Cancer Res 50:421-425, 1990
8. Wistuba II, Bryant D, Behrens C, et al: Comparison of features of human lung cancer cell lines and their corresponding tumors. Clin Cancer Res 5:991-1000, 1999
9. Schneider PM, Hung MC, Chiocca SM, et al: Differential expression of the c-erbB-2 gene in human small cell and non–small cell lung cancer. Cancer Res 49:4968-4971, 1989
10. Kern JA, Robinson RA, Gazdar A, et al: Mechanisms of p185HER2 expression in human non–small cell lung cancer cell lines. Am J Respir Cell Mol Biol 6:359-363, 1992
11. Tsai CM, Chang KT, Wu LH, et al: Correlations between intrinsic chemoresistance and HER-2/*neu* gene expression, p53 gene mutations, and cell proliferation characteristics in non–small cell lung cancer cell lines. Cancer Res 56:206-209, 1996
12. Cline MJ, Battifora H: Abnormalities of protooncogenes in non–small cell lung cancer. Correlations with tumor type and clinical characteristics. Cancer 60:2669-2674, 1987
13. Kern JA, Schwartz DA, Nordberg JE, et al: P185*neu* expression in human lung adenocarcinomas predicts shortened survival. Cancer Res 50:5184-5187, 1990
14. Sozzi G, Miozzo M, Tagliabue E, et al: Cytogenetic abnormalities and overexpression of receptors for growth factors in normal bronchial epithelium and tumor samples of lung cancer patients. Cancer Res 51:400-404, 1991
15. Harpole DH Jr, Marks JR, Richards WG, et al: Localized adenocarcinoma of the lung: Oncogene expression of erbB-2 and p53 in 150 patients. Clin Cancer Res 1:659-664, 1995
16. Guinee DG Jr, Travis WD, Trivers GE, et al: Gender comparisons in human lung cancer: Analysis of p53 mutations, anti-p53 serum antibodies and C-erbB-2 expression. Carcinogenesis 16:993-1002, 1995
17. Giatromanolaki A, Koukourakis MI, O'Byrne K, et al: Non–small cell lung cancer: c-erbB-2 overexpression correlates with low angiogenesis and poor prognosis. Anticancer Res 16:3819-3825, 1996
18. Visscher DW, Yadrandji S, Tabaczka P, et al: Clinicopathologic analysis of k-ras, p53, and ERBB-2 gene alterations in pulmonary adenocarcinoma. Diagn Mol Pathol 6:64-69, 1997
19. Volm M, Efferth T, Mattern J: Oncoprotein (c-myc, c-cerbB2, c-fos) and suppressor gene product (p53) expression in squamous cell carcinomas of the lung. Clinical and biological correlations. Anticancer Res 12:11-20, 1992
20. Shiraishi M, Noguchi M, Shimosato Y, et al: Amplification of protooncogenes in surgical specimens of human lung carcinomas. Cancer Res 49:6474-6479, 1989
21. Pastorino U, Andreola S, Tagliabue E, et al: Immunocytochemical markers in stage I lung cancer: Relevance to prognosis. J Clin Oncol 15:2858-2865, 1997
22. Gerdes AM, Nielsen O, Mohr U, et al: Correlation between molecular genetic analyses and immunohistochemical evaluation of the epidermal growth factor receptor and p185HER2. Anticancer Res 18:2529-2534, 1998
23. Kwiatkowski DJ, Harpole DH Jr, Godleski J: Molecular pathologic substaging in 244 stage I non–small cell lung cancer patients: Clinical implications. J Clin Oncol 16:2468-2477, 1998
24. Kim YC, Park KO, Kern JA, et al: The interactive effect of Ras, HER2, p53 and Bcl-2 expression in predicting the survival of non–small cell lung cancer patients. Lung Cancer 22:181-190, 1998
25. Slamon DJ, Clark GM, Wong SG, et al: Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science 235:177-182, 1987
26. Chou TC, Talalay P: Analysis of combined drug effects: A new look at a very old problem. Trends Pharmacol Sci 4:450-454, 1983
27. Slamon DJ, Godolphin W, Jones LA, et al: Studies of the HER-2/*neu* proto-oncogene in human breast and ovarian cancer. Science 244:707-712, 1989

28. Berchuck A, Kamel A, Whitaker R, et al: Overexpression of HER-2/*neu* is associated with poor survival in advanced epithelial ovarian cancer. Cancer Res 50:4087-4091, 1990

29. Scambia G, Panici PB, Ferrandina G, et al: Expression of HER-2/*neu* oncoprotein, DNA-ploidy and S-phase fraction in advanced ovarian cancer. Int J Gynaecol Cancer 3:271-278, 1993

30. Rubin SC, Finstad CL, Wong GY, et al: Prognostic significance of HER-2/*neu* expression in advanced epithelial ovarian cancer: A multivariate analysis. Am J Obstet Gynecol 168:162-169, 1993

31. Felip E, Del Campo JM, Rubio D, et al: Overexpression of c-erbB-2 in epithelial ovarian cancer: Prognostic value and relationship with response to chemotherapy. Cancer 75:2147-2152, 1995

32. Fajac A, Benard J, Lhomme C, et al: c-erbB2 gene amplification and protein expression in ovarian epithelial tumors: Evaluation of their respective prognostic significance by multivariate analysis. Int J Cancer 64:146-151, 1995

33. van der Zee AG, Hollema H, Suurmeijer AJH, et al: Value of P-glycoprotein, glutathione S-transferase pi, c-erbB-2, and p53 as prognostic factors in ovarian carcinomas. J Clin Oncol 13:70-78, 1995

34. van Dam PA, Vergote IB, Lowe DG, et al: Expression of c-erbB-2, c-myc, and c-ras oncoproteins, insulin-like growth factor receptor I, and epidermal growth factor receptor in ovarian carcinoma. J Clin Pathol 47:914-919, 1994

35. Pietras RJ, Fendly BM, Chazin VR, et al: Antibody to HER-2/*neu* receptor blocks DNA repair after cisplatin in human breast and ovarian cancer cells. Oncogene 9:1829-1838, 1994

36. Hancock MC, Langton BC, Chan T, et al: A monoclonal antibody against the c-erbB-2 protein enhances the cytotoxicity of cis-diamminedichloroplatinum against human breast and ovarian tumor cell lines. Cancer Res 51:4575-4580, 1991

37. Ozols RF: Update of the National Comprehensive Cancer Network ovarian cancer practice guidelines. Oncology NCCN Proc 11:95-105, 1997

38. Bookman MA, Birrer M, Karlan B, et al: A phase II evaluation of anti-HER-2/*neu* antibody (NSC #688097) (IND #6667) in recurrent or refractory ovarian or primary peritoneal carcinoma. Gynecologic Oncology Group Protocol #160

39. Gu K, Mes-Masson AM, Gauthier J, et al: Overexpression of HER-2/*neu* in human prostate cancer and benign hyperplasia. Cancer Lett 99:185-189, 1996

40. Ross JS, Sheehan C, Hayner-Buchan AM, et al: HER-2/*neu* gene amplification status in prostate cancer by fluorescence in situ hybridization. Hum Pathol 28:827-833, 1997

41. Sadasivan R, Morgan R, Jennings S, et al: Overexpression of HER-2/*neu* may be an indicator of poor prognosis in prostate cancer. J Urol 150:126-131, 1993

42. Ross JS, Sheehan CE, Hayner-Buchan AM, et al: Prognostic significance of HER-2/*neu* gene amplification status by fluorescence in situ hybridization of prostate carcinoma. Cancer 79:2162-2170, 1997

43. Kuhn EJ, Kurnot RA, Sesterhenn IA, et al: Expression of the c-erbB-2 (HER-2/*neu*) oncoprotein in human prostatic carcinoma. J Urol 150:1427-1433, 1993

44. Zhau H, Wan D, Zhou J, et al: Expression of c-erb B-2/*neu* proto-oncogene in human prostatic cancer tissues and cell lines. Mol Carcinog 5:320-327, 1992

45. Visakorpi T, Kallioniemi OP, Koivula T, et al: Expression of epidermal growth factor receptor and ERBB2 (HER-2/*neu*) oncoprotein in prostatic carcinomas. Mod Pathol 5:643-648, 1992

46. Agus DB, Scher HI, Higgins B, et al: Response of prostate cancer to anti-HER-2/*neu* antibody in androgen-dependent and independent human xenograft models. Cancer Res 59:4761-4764, 1999

47. Nagabhushan M, Miller CM, Pretlow TP, et al: CWR22: The first human prostate cancer xenograft with strongly androgen-dependent and relapsed strains both in vivo and in soft agar. Cancer Res 56:3042-3046, 1996

48. Wainstein MA, He F, Robinson D, et al: CWR22: Androgen-dependent xenograft model derived from a primary human prostatic carcinoma. Cancer Res 54:6049-6052, 1994

49. Stearns ME, Ware JL, Agus DB, et al: Workgroup 2: Human xenograft models of prostate cancer. Prostate 36:56-58, 1998

50. Craft N, Shostak Y, Carey M, et al: A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/*neu* tyrosine kinase. Nat Med 5:280-285, 1999

STIC-ILL

RD155.8.H85

From: Hunt, Jennifer
Sent: Tuesday, January 22, 2002 5:54 PM
To: STIC-ILL
Subject: References for 09/602,800

Please send me the following references ASAP:

Hum. Gene Ther. (1997), 8(2), 157-170

CLINICAL CANCER RESEARCH, (1999 Aug) 5 (8) 2171-7

CANCER RESEARCH, (1999 Oct 1) 59 (19) 4761-4

SEMINARS IN ONCOLOGY, (2000 Dec) 27 (6 Suppl 11) 53-63; discussion 92-100

SEMINARS IN ONCOLOGY, (2001 Aug) 28 (4 Suppl 15) 3-7

SEMINARS IN ONCOLOGY, (2001 Aug) 28 (4 Suppl 15) 71-6

Thanks,

Jennifer Hunt
Patent Examiner, Art Unit 1642
CM1-8D06 (mailbox 8E12)
(703)308-7548

HUMAN GENE THERAPY 8:157–170 (January 20, 1997)
Mary Ann Liebert, Inc.

A Novel Gene Therapy Strategy for Elimination of Prostate Carcinoma Cells from Human Bone Marrow

MYUNGHEE KIM,[1] MARCI WRIGHT,[1] JESSY DESHANE,[1] MARY ANN ACCAVITTI,[1] ARABELLA TILDEN,[2,3] MANSOOR SALEH,[2] WILLIAM P. VAUGHAN,[2,3] MATTHEW H. CARABASI,[2,3] MIRIAM D. ROGERS,[4] RICHARD D. HOCKETT, JR.,[4] WILLIAM E. GRIZZLE,[4] and DAVID T. CURIEL[1,4]

ABSTRACT

We report a novel means to purge bone marrow of a specific subset of prostate carcinoma cells based on transductional and genetic selectivity. Using both adenovirus–polylysine–DNA complexes and E1A/B-deleted replication-deficient adenoviruses, we have demonstrated a transductional preference of these vectors for the prostate carcinoma cell lines DU 145, LNCaP, and PC-3 over primary human bone marrow cells and the leukemia cell line KG-1. We have also shown a genetic selectivity of an anti-erbB-2 intracellular single-chain antibody (sFv) encoding adenovirus, Ad21, for the erbB-2-positive prostate carcinoma cell lines DU 145 and LNCaP. Delivery of Ad21 resulted in cytotoxicity to the DU 145 and LNCaP, but not PC-3, cell lines and reduced the clonogenic capacity of DU 145 cells cultured alone or mixed with various ratios of irradiated human bone marrow. Finally, quantitative, competitive reverse transcription polymerase chain reaction (QC-RT-PCR) analysis demonstrated that Ad21 could effectively reduce DU 145 and erbB-2-positive primary prostate tumor contamination in bone marrow cultures. Delivery of Ad21 had no effect on the ability of progenitor cells to form colonies. These results suggest that an anti-erbB-2 sFv-encoding adenoviral vector is efficacious for removal of erbB-2-positive prostate carcinoma cells from human bone marrow, and demonstrates a novel method for *ex vivo* genetic purge of malignant cells from bone marrow for autologous bone marrow transplantation (ABMT) therapy.

OVERVIEW SUMMARY

Chemotherapy and immunotherapy have been utilized to purge human bone marrow of tumor cell populations to improve the prognostic outcome of patients undergoing autologous bone marrow transplantation (ABMT). In this report, we show that adenovirus-mediated delivery of an anti-erbB-2 single-chain intracellular antibody (sFv) purges human bone marrow of erbB-2-positive prostate carcinoma cells. Both adenovirus and adenovirus–polylysine–DNA (AdpL) vectors encoding the luciferase and β-galactosidase (β-Gal) reporter genes preferentially transduced prostate carcinoma cell lines, but did not transduce either primary bone marrow cells or a leukemia cell line. The adenovirus encoding the anti-erbB-2 sFv was cytot xic to the erbB-2-positive prostate carcinoma cell lines, but not to hematopoietic progenitor cells, and was able to abrogate the clonogenicity of DU 145 prostate carcinoma cells mixed with irradiated bone marrow. Quantitative, competitive reverse transcription polymerase chain reaction (QC-RT-PCR) analysis showed that an erbB-2-positive prostate carcinoma cell line and primary prostate carcinoma disease were essentially eliminated from bone marrow cells when treated with the anti-erbB-2 sFv encoding adenovirus.

INTRODUCTION

AUTOLOGOUS BONE MARROW TRANSPLANTATION (ABMT) has been employed in a variety of neoplastic disease contexts to facilitate the administration of high-dose chemotherapy. In this regard, ABMT has become the accepted treatment modal-

[1]Gene Therapy Program, [2]Division of Hematology and Oncology, Department of Internal Medicine, [3]Bone Marrow Transplantation Program, [4]Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35294.

ity for selected groups of patients with malignant lymphomas and a variety of solid tumors including neuroblastoma and breast cancer (Peters *et al.*, 1993; Bociek *et al.*, 1995). Our group has also begun to explore the utility of this approach for advanced carcinoma of the prostate.

To eliminate the possibility of reinfusion of malignant cells, ABMT strategies have been coupled to a variety of methods for eliminating tumor cells from the marrow or peripheral blood stem cells (PBSC) (Gribben and Nadler, 1993; Gee, 1995). Current marrow "purging" approaches have employed mechanical, chemical, and immunological procedures (Kies *et al.*, 1988; Meyer *et al.*, 1991; Myklebust *et al.*, 1994; Moriyama *et al.*, 1992; Juneja *et al.*, 1995). Whereas these various methodologies may achieve significant reductions in tumor cell contamination of marrow, these procedures are generally highly complex and labor intensive. In addition, the utility of these methods is contingent upon high-level expression of cancer-associated cell-surface markers (Gross *et al.*, 1995; Simpson *et al.*, 1995). We report here a means to achieve marrow purge employing a novel gene therapy method. Our strategy is based upon a vector delivery approach, which accomplishes preferential transduction of tumor cells, coupled with a mutation compensation strategy, to achieve selective cytotoxicity in tumor cells. Employing this combination approach, tumor cells can be effectively eliminated without significant reduction in viability of bone marrow progenitor cells. This procedure may offer significant advantages over described methods in terms of feasibility and applicability to a wide range of bone marrow purge contexts.

MATERIALS AND METHODS

Bone marrow

Human bone marrow consisting of residual material from normal marrow processed for transplantation was obtained from the University of Alabama at Birmingham Hematopoietic Stem Cell Processing Facility and used throughout this study. Marrow cells were separated by Ficoll-Hypaque centrifugation at 900 × *g* for 20 min. Mononuclear cells were harvested from the interface, washed twice in phosphate-buffered saline (PBS, pH 7.2), and cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 2% fetal bovine serum (FBS) (StemCell Technologies, Inc., Vancouver, BC, Canada). Cells were irradiated with 5,000 rads prior to use in clonogenic mixing experiments with prostate carcinoma cells.

Bone marrow aspirate

An aspirate consisting of bone marrow cells contaminated with primary erbB-2-positive prostate carcinoma cells was obtained frozen from the Hematopoietic Stem Cell Processing Facility. Cells were quick-thawed at 37°C, resuspended in IMDM, and incubated at room temperature with 10 mg of DNase I (Sigma Corp., St. Louis, MO) for 1 min. Cells were then washed once with IMDM and equilibrated at room temperature for 2 hr. Mononuclear cells were harvested from the aspirate as described above and used the same day in quantitative reverse transcription polymerase chain reaction (QC-RT-PCR) experiments.

Cell lines

The human prostate carcinoma cells lines DU 145, LNCaP, PC-3, the human cervical carcinoma cell line HeLa, and the human bone marrow leukemia cell line KG-1 were obtained from the American Type Culture Collection (Rockville, MD). Prostate carcinoma cell lines were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS) (PAA, Newport Beach, CA), L-glutamine (300 μg/ml), penicillin (100 IU/ml), and streptomycin (25 μg/ml). HeLa cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) with the aforementioned supplements. The KG-1 cells were cultured in IMDM.

Gene transfer vectors

Reporter plasmids encoding either *Escherichia coli* β-galactosidase (pCMVβ) or firefly luciferase (pcLUC4) were combined with adenovirus-polylysine conjugate (AdpL) as previously described (Deshane *et al.*, 1994). Replication-incompetent, E1A/B-deleted recombinant adenoviruses encoding either the β-Gal gene (AdCMVLacZ) or the luciferase gene (AdCMVLuc) were kindly provided by Dr. De-chu Tang (University of Alabama at Birmingham, Birmingham, AL) and Dr. Robert Gerard (University of Texas, Dallas, TX), respectively. An adenovirus encoding the endoplasmic reticulum (ER) directed anti-erbB-2 intracellular single-chain antibody (sFv) (Ad21) used for inducing specific cytotoxicity in tumor cells has been previously described (Deshane *et al.*, 1995b; Grim *et al.*, 1996). Adenoviral vectors were delivered to the target cells at a multiplicity of infection (moi) of 100 plaque-forming units (PFU) per cell. Cells were transduced with either the adenovirus (Ad) or AdpL vector in reduced serum medium (either RPMI-1640 or IMDM with 2% FCS) for 1 hr, then supplemented with appropriate culture medium and maintained until ready for analysis.

Reporter gene assays

Cells seeded into six-well tissue culture plates at about 70% confluency were transduced with the firefly luciferase or *E. coli* β-Gal reporter genes via the AdpL vector or the recombinant adenovirus. At 24 hr post-infection, cell lysates were assayed for luciferase expression with a commercial luciferase assay kit (Promega Corp., Madison, WI) employing manufacturer's recommendations. At 48 hr post-infection, β-Gal expression was determined by flow cytometric methods using cells stained with fluorescein-di-β-D-galactopyranoside (FDG) (Sigma, St. Louis, MO). Briefly, cells were suspended at a concentration of 1×10^7 cells/ml in staining medium (PBS with 4% FCS and 10 m*M* HEPES). An aliquot of cells (100 μl) was incubated at 37°C for 10 min and stained with 100 μl of prewarmed 200 m*M* FDG for 1 min. The reaction was quenched by the addition of 1 ml of staining medium, and cells were immediately assayed for FDG uptake by fluorescence-activated cell sorting (FACS) analysis.

Cell viability assay

Cells were seeded at a density of 5×10^3 cells per well in 96-well plates (Costar, Cambridge, MA). Twenty-four hours later, adenoviral vectors were delivered to the cells as described

above. At 96 hr post-infection, direct analysis of cell viability was measured using the Cell Titer 96 AQ Non-Radioactive Cell Proliferation Assay (Promega Corp. Madison, WI). This assay is based on the ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) to a formazan that is soluble in tissue culture medium and can be measured spectrophotometrically at an absorbance of 490 nm. MTS solution (2 ml) was mixed with 100 μl of phenazine methosulfate (PMS) immediately before addition to the cells in the culture plate. MTS/PMS solution (20 μl) was then added into each well, maintaining a rate of 20 μl of MTS/PMS to 100 μl of medium. After 30 min, the reduction product was measured at an absorbance of 490 nm and compared to a standardized curve.

Colony-forming unit assay

All colony-forming unit-granulocyte erythroid monocyte macrophage (CFU-GEMM) assays were performed in triplicate. Fresh bone marrow cells were transduced with a control virus (AdCMVLacZ) or an adenovirus encoding the endoplasmic recticulum (ER) directed anti-erbB-2 sFv (Ad21) at 100 pfu/cell. One hour post-transduction, a total of 6×10^5 cells were resuspended in 3 ml of Iscove's methylcellulose medium supplemented with 10% agar leukocyte conditioned medium, 30% FBS, erythropoietin, bovine serum albumin (BSA), 2-mercaptoethanol, and L-glutamine (StemCell Technologies, Inc., Vancouver, BC, Canada). The suspension was plated onto 35-mm gridded dishes and colonies of >12 cells were scored 25 days post-infection.

Clonogenic assay

All clonogenic assays were performed in triplicate. The DU 145 cell line was either treated alone, or mixed with irradiated bone marrow cells at ratios of 1%, 10%, and 50% DU 145 cells. The target cells were transduced with a control virus (AdCMVLuc) or an adenovirus encoding the ER-directed anti-erbB-2 sFv (Ad21) at 100 pfu/cell. A total of 1×10^4 cells were then resuspended in 1 ml of 2× RPMI-1640 supplemented with 20% FCS, L-glutamine, penicillin, streptomycin, and 0.2% noble agar (DIFCO, Detroit, MI). The cell suspension was plated over a layer of 0.4% noble agar in 1 ml of 2× RPMI-1640 supplemented with 20% FCS, L-glutamine, penicillin, and streptomycin in 35-mm gridded dishes. A linear dilution of untransduced prostate cells was also plated at 1×10^2 cells/plate to 5×10^4 cells/plate to determine cloning efficiency. Colonies of >12 cells were scored 21 days post-infection.

Immunohistochemistry

Paraffin sections (5 μm) of prostate carcinoma tissue resected from a patient were deparaffinized in xylene and rehydrated prior to immunostaining. Slides were treated with 3% H_2O_2 for 5 min to quench endogenous peroxidase activity and incubated with preimmune goat serum (1%) for 1 hr to decrease nonspecific staining. The sections were then incubated with a monoclonal antibody specific for human $p185^{erbB-2}$, clone 3B5 (Oncogene Science, Uniondale, NY), at a concentration of 0.25 μg/ml for an hour at room temperature. An irrelevant mouse immunoglobulin G (IgG) (5 μg/ml) was used as a control for monoclonal antibody specificity. A commercial secondary detection system (Biogenex, San Ramon, CA) was used according to manufacturer's instructions for visualization of the erbB-2 protein. The slides were counterstained with hematoxylin.

Quantitative, competitive RT-PCR

A bone marrow aspirate contaminated with primary erbB-2-positive prostate carcinoma cells or fresh donor bone marrow spiked with 1% DU 145 prostate carcinoma cells were seeded in six-well plates at 1×10^5 cells/well. The cells were transduced with the ER-directed anti-erbB-2 sFv encoding adenovirus, Ad21, at 100 PFU/cell. Untransduced cells were used as a control. At 1, 2, 3, and 7 days post-infection, RNA isolation and extraction were carried out on the cells essentially as described (Hockett *et al.*, 1995). Cells were lysed in 4 *M* guanadine solution at ~1,000 cells per microliter and stored at −80°C. A total of 2,000–5,000 cells were typically used per titration point. Combination of cell extracts with competitors, and the performance of reverse transcription (RT) and the polymerase chain reaction (PCR) were as described (Hockett *et al.*, 1995), except isolated RNAs were treated with the denaturant methyl mercury hydroxide (MeHgOH) prior to RT. Following MeHgOH, RT was performed at 50°C with Superscript II (GIBCO-BRL, Gaithersburg, MD). The primer template for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH) is contained in the competitor pQPCR.GFO1.2 and for erbB-2; the template is contained in competitor pQPCR.BRC1 (data not shown). Primers for G3PDH were 5′ sense primer 5′-TCCTGCACCACCAACTG and 3′ antisense primer 5′-BIOTIN-GCCTGCTTCACCACCTT. For erbB-2, the primers were: 5′ sense primer 5′-CTGGTGCCACTCTGGAA and 3′ antisense primer 5′-BIOTIN-CTCAAGCAGGAAGGAAG. PCR conditions were 94°C for 30 sec, 55°C for 1 min, and 72°C for 30 sec for 35 cycles. PCR reactions for G3PDH had a final Mg^{2+} concentration of 2.6 m*M* and erbB-2 reactions had a final concentration of 1.6 m*M*. Plate-based EIA of PCR products was performed as described (Hockett *et al.*, 1995). The detection oligonucleotide for G3PDH was 5′-CCTGACCTGCCGTCTAGAAAAACCT and for erbB-2 was 5′-GCCCAGCCTTCGACAACCTCTATTA.

RESULTS

Selective transduction of human prostate carcinoma cells

To accomplish selective elimination of carcinoma cells from marrow by a gene therapy approach, viral vectors were evaluated for their potential to achieve preferential gene delivery to tumor targets at high efficiency. In this regard, effective transduction of a variety of tumor targets has been reported employing recombinant adenoviral vectors (Brody *et al.*, 1994; Colak *et al.*, 1995; Deshane *et al.*, 1995b; Feng *et al.*, 1995). In addition, a vector approach based upon the derivation of adenovirus–polylysine–DNA (AdpL) complexes has been developed and its efficacy shown in accomplishing efficient gene delivery to various carcinoma cells (Curiel *et al.*, 1992). Thus, the utility of these two vector approaches for transduction of three human prostate carcinoma cell lines was analyzed. For the initial studies of vector efficiency, firefly luciferase was employed

as a reporter gene. This analysis provided an index of net gene expression based upon transduction efficacy of target cells. A replication-incompetent adenoviral vector encoding the firefly luciferase gene (AdCMVLuc) was used at an moi of 100 pfu per cell to transduce the various cellular targets. The cell lysates were then analyzed for expression of the luciferase gene 24 hr later using the human cervical carcinoma cell line HeLa, a cell line reported to be highly transducible by both candidate vectors (Deshane *et al.*, 1994; Rosenfeld *et al.*, 1995), as a positive control. Figure 1A shows that the human prostate carcinoma cell lines DU 145 and LNCaP expressed luciferase at levels comparable to those observed in the highly transducible HeLa cell line. The human prostate cell line PC-3 also expressed high levels of the reporter gene, although not of the same magnitude as the other prostate tumor targets. The AdpL vector system also accomplished high levels of luciferase gene expression in these cell lines (Fig. 1B). For these experiments, AdpL–DNA complexes that contained the luciferase-encoding expression plasmid pcLUC4 were prepared. As before, the DU145 and LNCaP lines exhibited higher levels of reporter gene expression than the PC-3 line. In contrast to the results obtained with the adenoviral vector, overall levels of reporter gene expression in the various human prostate carcinoma cell lines were somewhat less than that observed with the HeLa control.

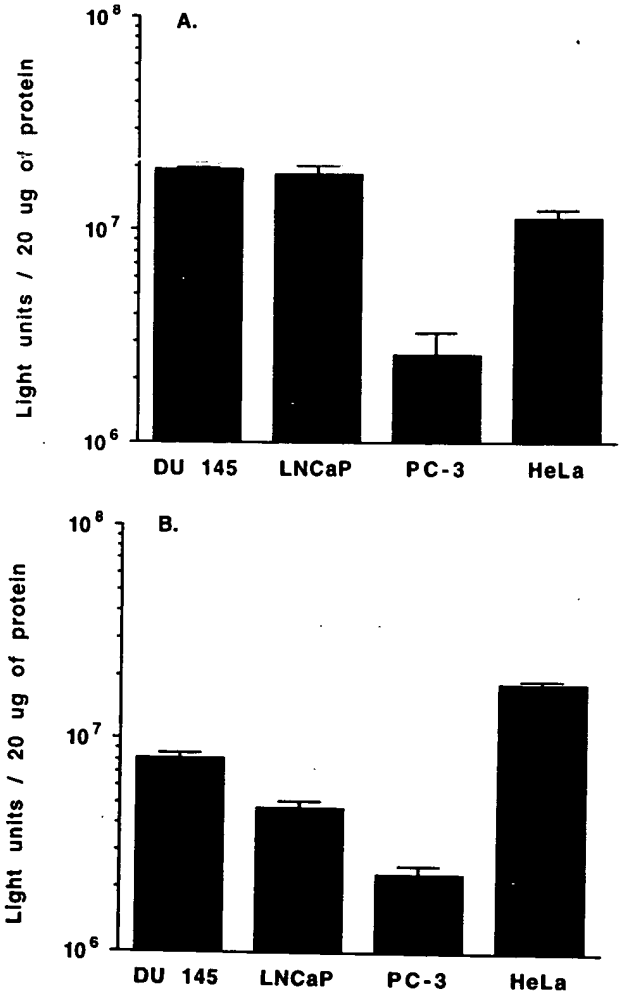


FIG. 1. Relative transduction efficiency of recombinant adenovirus and AdpL complexes for human prostate carcinoma cells. Three human prostate carcinoma cell lines were transduced with either of two vectors encoding the firefly luciferase reporter gene, and 24 hr post-transduction cellular lysates were analyzed for luciferase expression. Vectors included the E1A/B-deleted replication-incompetent luciferase-encoding adenovirus, AdCMVLuc, and AdpL complexes containing the luciferase-encoding plasmid pcLUC4. HeLa, a human cervical carcinoma cell line, was used as a positive control. A. Transduction efficiency of recombinant adenovirus. B. Transduction efficiency of AdpL complexes. Experiments were performed three times with results reported as mean ± SEM.

In addition, quantitation of the degree of transduction of human prostate carcinoma cells achieved by these two vector systems was determined by FACS analysis. A replication-incompetent adenovirus encoding the *LacZ* gene (AdCMVLacZ) or AdpL complexes incorporating an expression plasmid encoding the *LacZ* gene (pCMVβ) were employed in this study. Target cells were transduced and analyzed for transduction frequency by FDG staining for detection of the *LacZ* product followed by FACS analysis. As seen in Fig. 2, A and B, target cells were effectively transduced as evidenced by the derivation of a distinct cell subpopulation expressing the *LacZ* gene. Quantification of the results provided a direct index of the percentage of transduced cells expressing the reporter gene (Table 1). In this study, both vectors systems achieved a highly efficient transduction of all of the human prostate carcinoma cell lines tested. Furthermore, HeLa control cells were transduced at >95% frequency, indicating the integrity of the transduction process. The DU 145, LNCaP, and PC-3 lines also demonstrated transduction frequencies of >95% in each instance. Although lower levels of net gene transfer were observed for the PC-3 line with the luciferase-encoding adenoviral vector, overall transduction frequency was nonetheless comparable to the other prostate carcinoma lines when quantitated by β-galactosidase (β-Gal) expression. This data implies that extremely high levels of transduction can be achieved employing both the adenoviral vector and the AdpL vector for prostate carcinoma cells.

Inefficient transduction of human bone marrow cells

Because a component of this strategy is based upon the concept that candidate vectors will preferentially transduce prostate carcinoma cells, experiments to determine if these vectors would also be relatively ineffective in accomplishing gene transfer to normal bone marrow targets were undertaken. The luciferase-encoding adenoviral vector AdCMVLuc was delivered to freshly isolated human bone marrow cells using HeLa cells and the human prostate carcinoma cell line DU 145 as controls. In this analysis, it was demonstrated that the levels of reporter gene expression in the fresh bone marrow cells were more than three orders of magnitude less than that observed with the human prostate carcinoma cell line DU 145 (Fig. 3A). For the AdpL complexes, a similar differential was seen (Fig. 3B).

Consistent results were observed when the transduction frequency of the bone marrow cells was evaluated using the *LacZ* reporter gene system. Figure 4, A and B, shows that a trans-

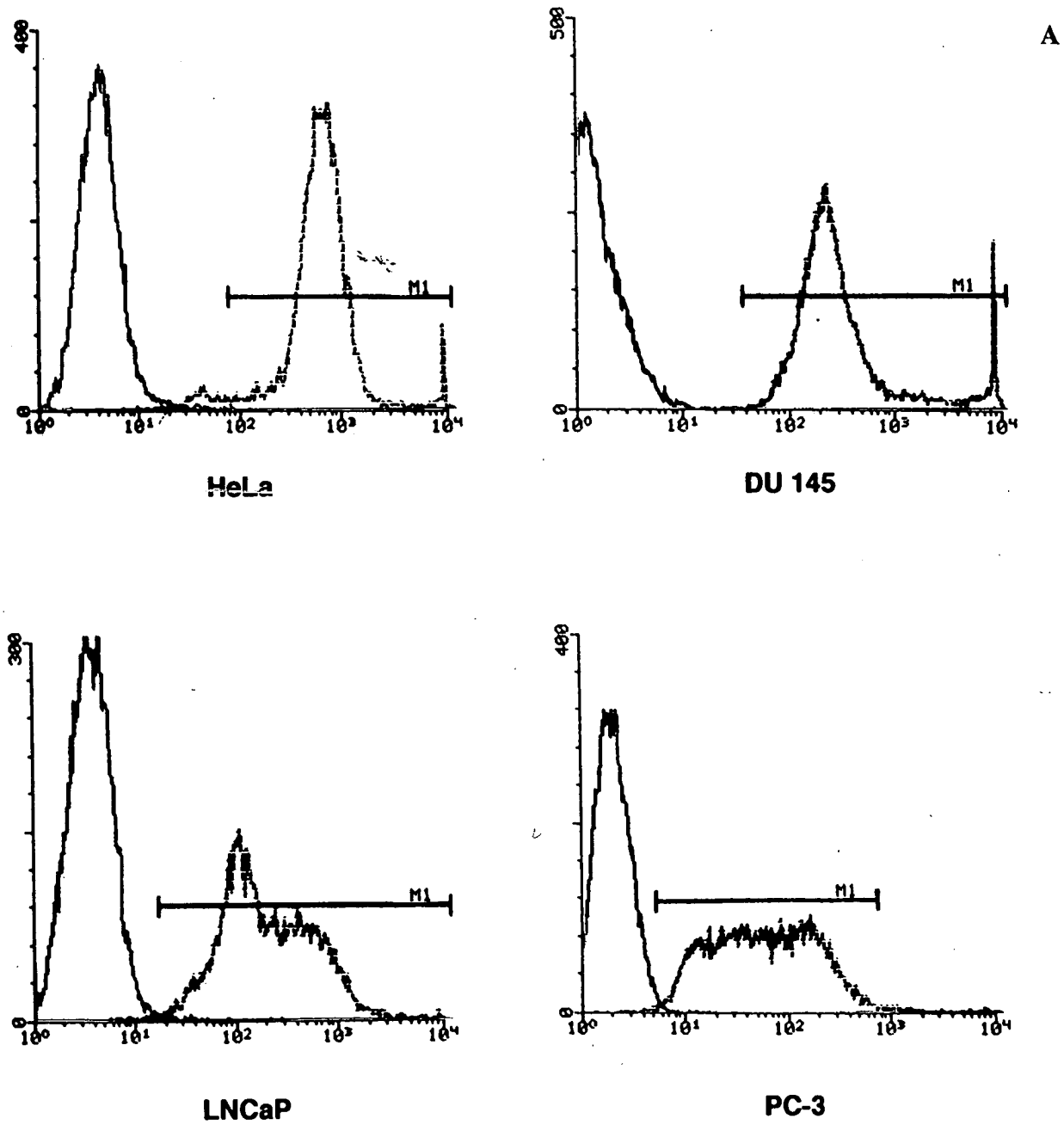


FIG. 2. Quantitative transduction efficiency of recombinant adenovirus and AdpL complexes for human prostate carcinoma cells. Three human prostate carcinoma cell lines were transduced with either of two vectors encoding the *E. coli* β-Gal gene (*LacZ*). Forty-eight hours post-transduction, cells were analyzed for expression of the *LacZ* gene after staining with FDG and performing FACS analysis. Vectors included the E1A/B-deleted, replication-incompetent *LacZ*-encoding adenovirus AdCMVLacZ and AdpL complexes containing the *LacZ*-encoding plasmid pCMVβ. HeLa, a human cervical carcinoma cell line, was used as a positive control. A. Quantitative transduction efficiency of recombinant adenovirus. B. Quantitative transduction efficiency of AdpL complexes.

duced subset of bone marrow cells could not be identified employing either the adenoviral vector or the AdpL complex. These data imply that adenoviral or AdpL vector systems do not accomplish efficient gene transfer to human bone marrow cells. The restricted vector tropism profile presented herein is consistent with the intended use of these reagents in the context of a marrow purge strategy.

Cytotoxic effect of anti-erbB-2 sFv encoding adenovirus on erbB-2 positive prostate carcinoma cell lines

The second selective component of this combination purging strategy employs the anti-erbB-2 sFv approach to tumor abrogation. Previous studies have shown that intracellular ex-

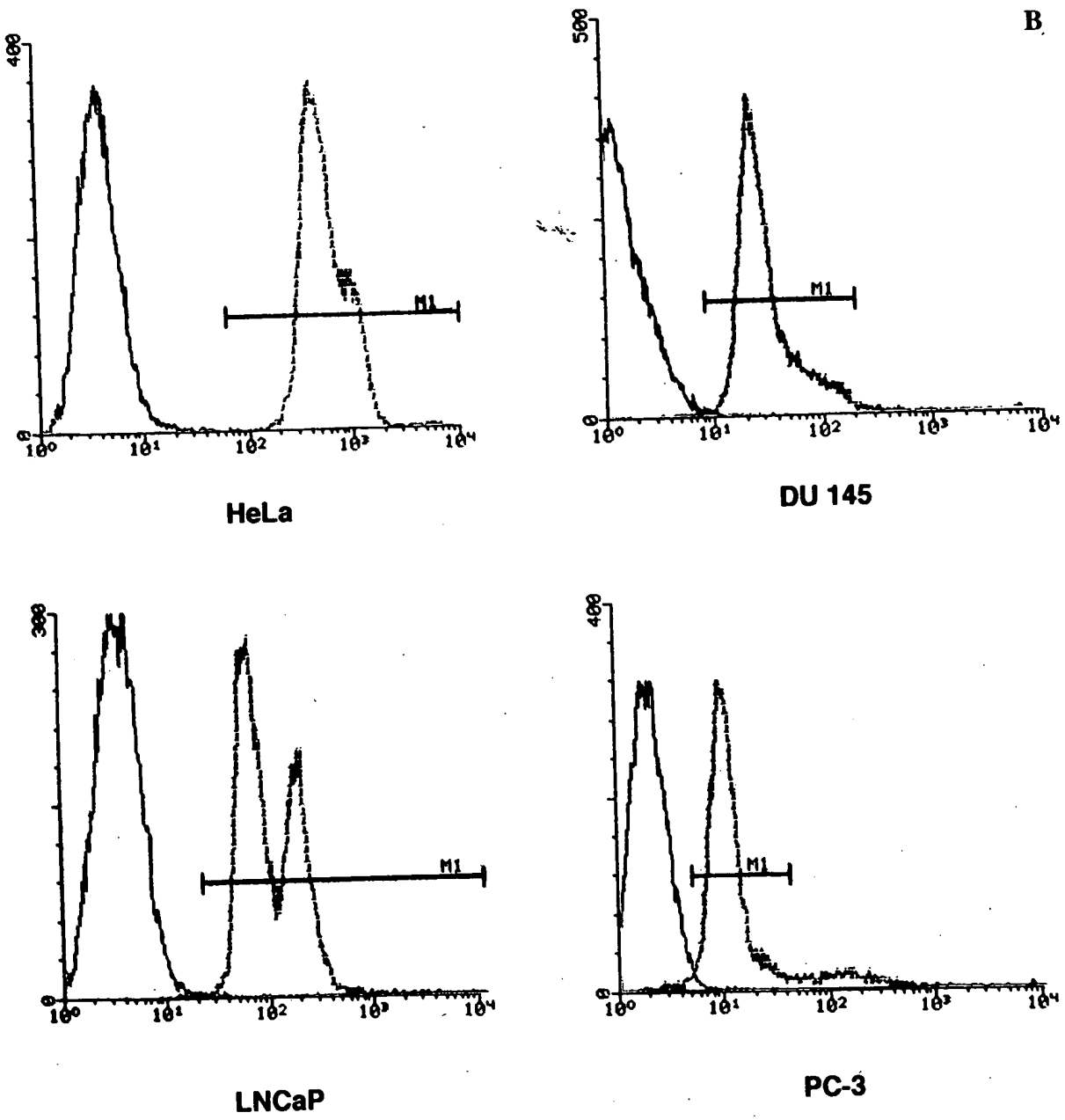


FIG. 2. (*Continued*)

pression of an anti-erB-2 sFv can induce cytotoxicity selectively in erbB-2-positive tumor targets (Deshane *et al.*, 1994; Deshane *et al.*, 1995a,b; Grim *et al.*, 1996). To validate the efficacy of anti-erbB-2 sFv-mediated cytotoxicity for prostate carcinoma cells in the context of an adenoviral vector, the E1A/B-deleted, replication-incompetent recombinant adenovirus encoding an ER-directed form of the anti-erbB-2 sFv (Ad21) was delivered to various prostate cancer cell lines. The luciferase-encoding adenoviral vector AdVCMVLuc was used as a control. Target cells for this analysis included human prostate carcinoma cell lines that have previously been shown to be erbB-2-positive (DU 145, LNCaP) or erbB-2-negative (PC-3) (Myers *et al.*, submitted). As noted earlier, each of these cell lines was transduced at >95% frequency by an adenoviral vector (Fig. 2A). The various cell lines were infected with Ad21 and analyzed for viability 96 hr post-transduction. Figure 5 illustrates that the anti-erbB-2 sFv-encoding adenovirus accomplished effective eradication of erbB-2-positive prostate cell lines DU 145 and LNCaP without cytotoxicity in the erbB-2-negative prostate carcinoma cell line, PC-3. Thus, the strategy of an intracellular antibody knockout appears to allow for selective tumor cell toxicity in the context of erbB-2-positive prostate tumor targets. In contrast, delivery of the control adenoviral AdCMVLuc at the same moi was not associated with any cytotoxicity in any of the human prostate carcinoma cell lines, suggesting that virus-associated nonspecific toxicity was not the basis of the observed antitumor effect. These data establish that adenoviral vector-mediated delivery of the anti-erbB-2 sFv can induce spe-

TABLE 1. QUANTITATIVE TRANSDUCTION EFFICIENCY OF RECOMBINANT ADENOVIRUS AND ADENOVIRUS-POLYLYSINE–DNA COMPLEXES FOR HUMAN PROSTATE CARCINOMA CELLS

	Transduction frequency (%)	
Cell line	*AdCMVLacZ*	*AdpL*
HeLa	96.19	99.99
DU 145	99.83	98.09
LNCaP	99.66	99.70
PC-3	98.08	87.70

cific cytotoxicity in erbB-2-positive prostate tumors based on genetic selectivity.

Noncytotoxic effect of anti-erbB-2 sFv-encoding adenovirus on human bone marrow

To confirm that Ad21 transduction does not kill non-erbB-2-expressing targets, the Ad21 vector was also applied to human bone marrow cells. Both the bone marrow-analogous human cell line KG-1, as well as fresh human bone marrow cells were used as targets in this experiment. The marrow cells were treated with the adenoviral vectors at an moi of 100 pfu per cell and then analyzed for viability at 96 hr post-transduction. Figure 5 indicates that the Ad21 vector did not induce any detectable cytotoxicity in KG-1 cells nor in fresh bone marrow cells. As an additional test of specificity, viral moi's were incrementally increased and bone marrow cells analyzed for viability as before. In this analysis, moi's of up to 1,000 pfu also did not cause any significant toxicity in the marrow cells (Fig. 6). The cell viability results were comparable for both the anti-erbB-2 sFv-encoding adenovirus Ad21 as well as the control adenovirus AdCMVLuc. In this instance, the specificity of the sFv-induced cytotoxicity exclusively in erbB-2-positive cells would not be predicted to be operative in the context of erbB-2-negative bone marrow cells. Taken together, these data strongly imply that the delivery of Ad21 was not cytotoxic to bone marrow cell viability.

Effect of anti-erbB-2 sFv-encoding adenovirus on bone marrow progenitor cell viability

To determine whether delivery of the anti-erbB-2 sFv-encoding adenovirus affected the ability of primary bone marrow progenitor cells to form colonies, colony-forming unit-granulocyte erythroid monocyte macrophage (CFU-GEMM) assays were performed. For this study, fresh human bone marrow cells were transduced with either a control adenovirus (AdCMVLacZ) or the anti-erbB-2 sFv-encoding adenovirus (Ad21) at 100 pfu per cell. The number of colonies formed by bone marrow cells transduced with the control adenovirus AdCMVLacZ were the same as those formed by untransduced bone marrow cells (Fig. 7A). The results demonstrate that exposure to adenoviral vectors is not deleterious to progenitor cells, thus affirming the relative inability of adenoviral vectors to transduce bone marrow. In addition, transduction of the bone marrow cells with Ad21 resulted in no change in the number of colonies formed as compared to untransduced, or AdCMVLacZ-transduced, progenitor cells. Therefore, transduction and/or expression of anti-erbB-2 sFv is not harmful to the non-erbB-2-expressing bone marrow cells.

Anti-erbB-2 sFv-mediated inhibition of anchorage-independent growth of an erbB-2-positive prostate carcinoma cell line

To determine the capability of this purging strategy to accomplish eradication of clonogenic prostate carcinoma cells, the Ad21 vector was delivered to the erbB-2-positive human prostate carcinoma cell line DU 145 with subsequent analysis for soft agar colony formation. Treatment of the prostate cells with the control adenovirus AdCMVLuc did not result in a

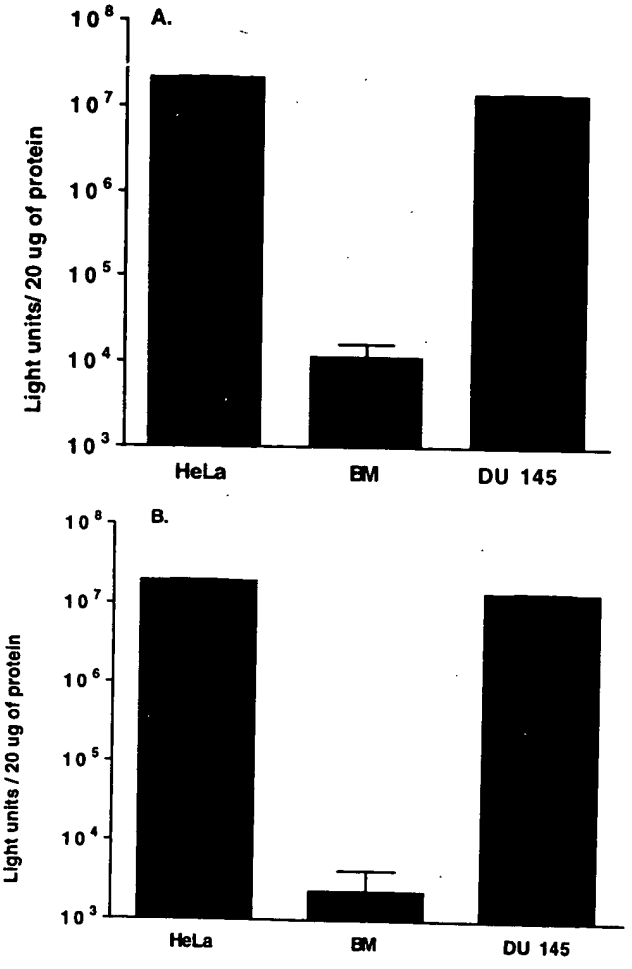


FIG. 3. Relative transductional efficacy of recombinant adenovirus and AdpL complexes for human bone marrow cells. Target cells were transduced with luciferase-encoding reporter genes *via* the recombinant adenovirus AdCMVLuc or AdpL complexes containing the plasmid, pcLUC4. Twenty-four hours post-transduction, cellular lysates were normalized for total protein content and analyzed for luciferase expression. Cells transduced included the human cervical carcinoma cell line HeLa, the human prostate carcinoma cell line DU 145, and fresh bone marrow cells derived from patient material. A. Transduction efficacy of recombinant adenovirus. B. Transduction efficacy of adenovirus–polylysine complexes. Experiments were performed three times with results reported as mean ± SEM.

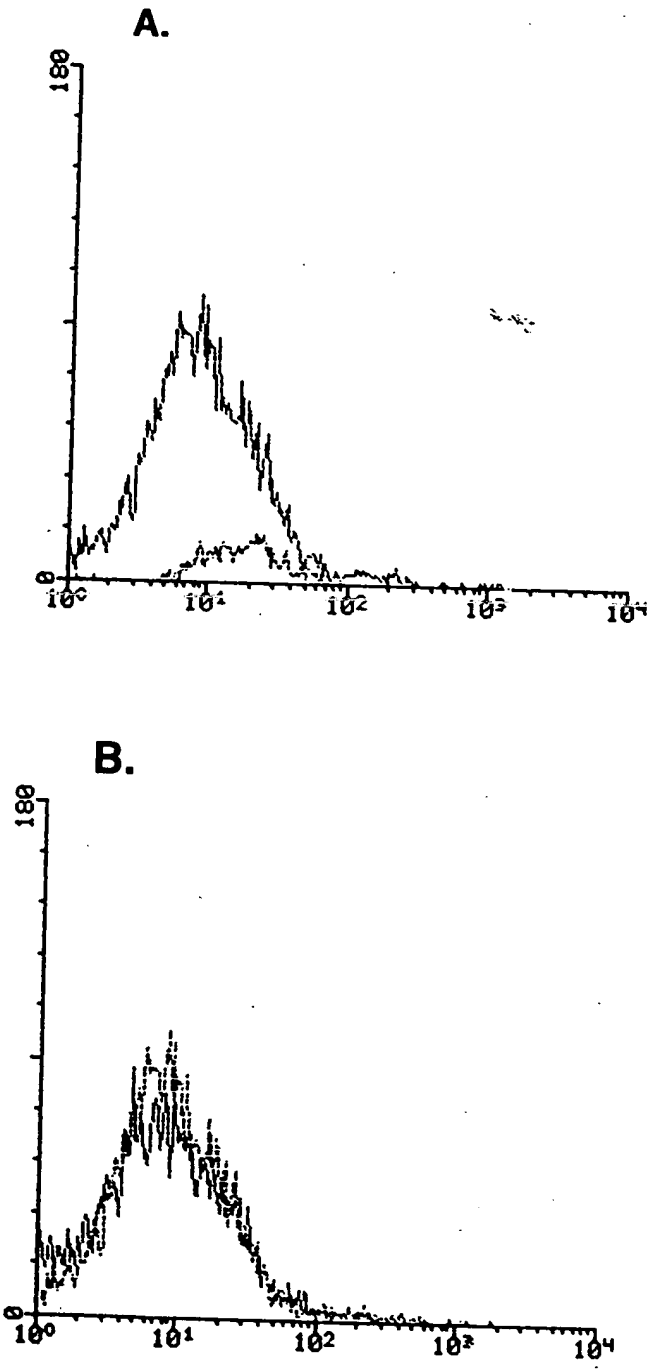


FIG. 4. Quantitative transduction efficiency of recombinant adenovirus and AdpL complexes for human bone marrow cells. Fresh bone marrow cells were transduced with *LacZ*-encoding reporter genes with either the recombinant adenovirus AdCMVLacZ or AdpL complexes containing the plasmid pCMVβ. Forty-eight hours post-transduction, bone marrow cells were analyzed for expression of the *LacZ* gene after staining with FDG and performing FACS analysis. A. Transduction efficiency of recombinant adenovirus. B. Transduction efficiency of AdpL complexes.

decrement of clonogenic cells compared to untransfected prostate carcinoma cells (Fig. 7B). In contrast, delivery of Ad21 resulted in a dramatic decrement in the number of colonies observed. As the viral vectors were delivered at a comparable moi, Fig. 7B reflects the specific antitumor effect of the anti-erbB-2 sFv.

To assess the ability of this methodology to eliminate tumor cells from fresh marrow, mixing experiments using the erbB-2-positive human prostate carcinoma cell line DU 145 and fresh, irradiated human bone marrow cells were investigated. At various tumor:bone marrow ratios, mixed cell populations were treated with the Ad21 vector and analyzed for clonogenicity of the tumor cell subset. Control experiments using untransduced DU 145 cells had established a cloning efficiency in the linear range for the tumor cell magnitudes studied (data not shown). Treating mixed marrow with the Ad21 anti-erbB-2 sFv-encoding adenovirus accomplished a significant reduction in tumor cells at all tested ratios compared to the control adenovirus, AdCMVLuc (Fig. 8). At a 50% dilution, Ad21 accomplished greater than two orders of magnitude decrement in clonogenic tumor cells, suggesting that once optimized, adenoviral vector delivery of the anti-erbB-2 sFv can effectively reduce the number of tumor cells in the context of a marrow purging approach.

Reduction of erbB-2 mRNA from bone marrow as determined by quantitative, competitive RT-PCR analysis

As an alternative methodology for determining degree of marrow purging quantitative, competitive RT-PCR (QC-RT-PCR) was used to analyze erbB-2 expression post-Ad21 treatment of marrow mixing experiments. In these assays, fresh bone marrow was mixed with 1% DU 145 cells, treated with Ad21, and then analyzed at 1, 2, and 7 days post-transduction by QC-RT-PCR for total erbB-2 mRNA content. As indicated in Fig. 9, a >99.9% reduction of erbB-2 mRNA following treatment with Ad21 over 7 days could be demonstrated by this analysis. These data closely agree with the clonogenic assays demonstrating effective elimination of erbB-2 positive carcinoma cells transduced with the anti-erbB-2 sFv-encoding adenovirus.

To evaluate the efficacy of the anti-erbB-2 sFv strategy to purge primary erbB-2-positive cells from bone marrow, an aspirate was obtained from a patient with documented metastasis of refractory prostate carcinoma. A section of the prostate biopsy from the same patient showed fields with strong immunoreactivity for erbB-2, as illustrated in Fig. 10. Additionally, quantitative measurements of erbB-2 mRNA were obtained from the primary aspirate. The cells were treated with Ad21 and analyzed for total erbB-2 mRNA content at 1, 2, and 3 days post-transduction. As shown in Fig. 11, a significant reduction in erbB-2 mRNA was seen, although complete elimination was not achieved in this case. Nevertheless, this purge strategy appears to function for primary tumor and marrow material derived in the context of clinical metastatic disease.

DISCUSSION

The defined strategy for marrow purge outlined herein is based upon vector selectivity at both the transductional as well as the genetic level. Adenoviral tropism for epithelial-derived cells allowed relative vector specificity for tumor targets within the context of the normal bone marrow progenitors. Therefore,

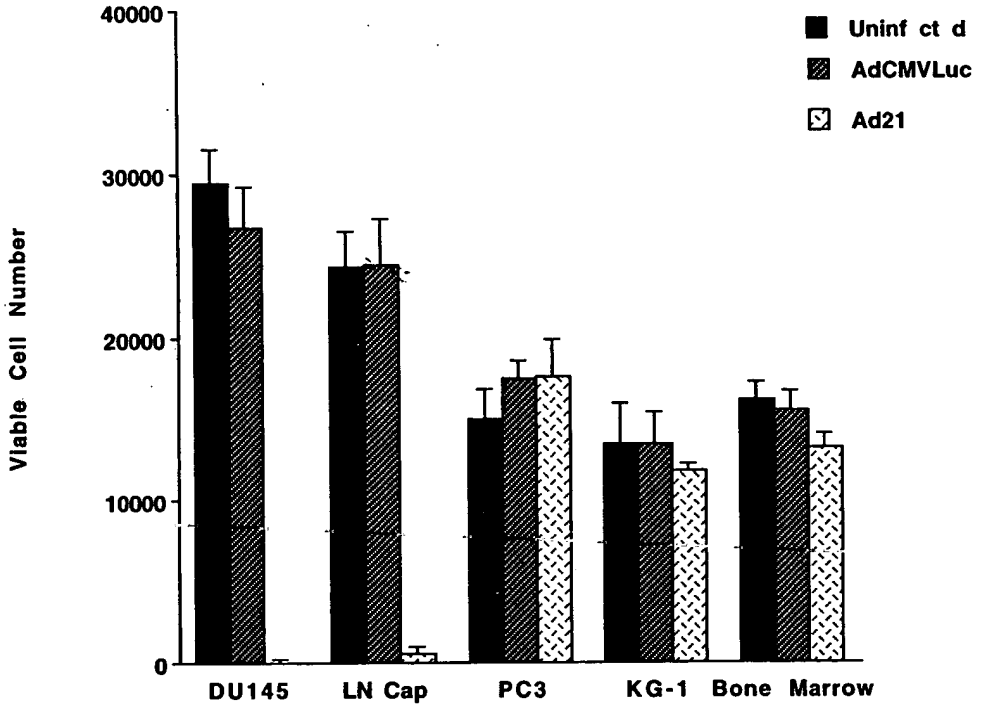


FIG. 5. Selective cytotoxicity of recombinant adenovirus encoding single-chain anti-erbB-2 antibody (sFv) directed for erbB-2-positive cells but not for human bone marrow cells. Human prostate carcinoma cell lines and human bone marrow cells were transduced with either a reporter gene encoding adenovirus (AdCMVLuc) or an adenovirus encoding the anti-erbB-2 (Ad21) and analyzed for viability by MTS assay 96 hr post-transduction. Untransduced cells served as an additional control. Target cells included erbB-2-positive human prostate carcinoma cell lines DU 145 and LNCaP and the erbB-2-negative human prostate cell line PC-3, as well as the leukemia cell line KG-1 and fresh human bone marrow. Adenoviral infections were carried out at a moi of 100 viral pfu per cell. Experiments were performed five times with results reported as mean ± SEM.

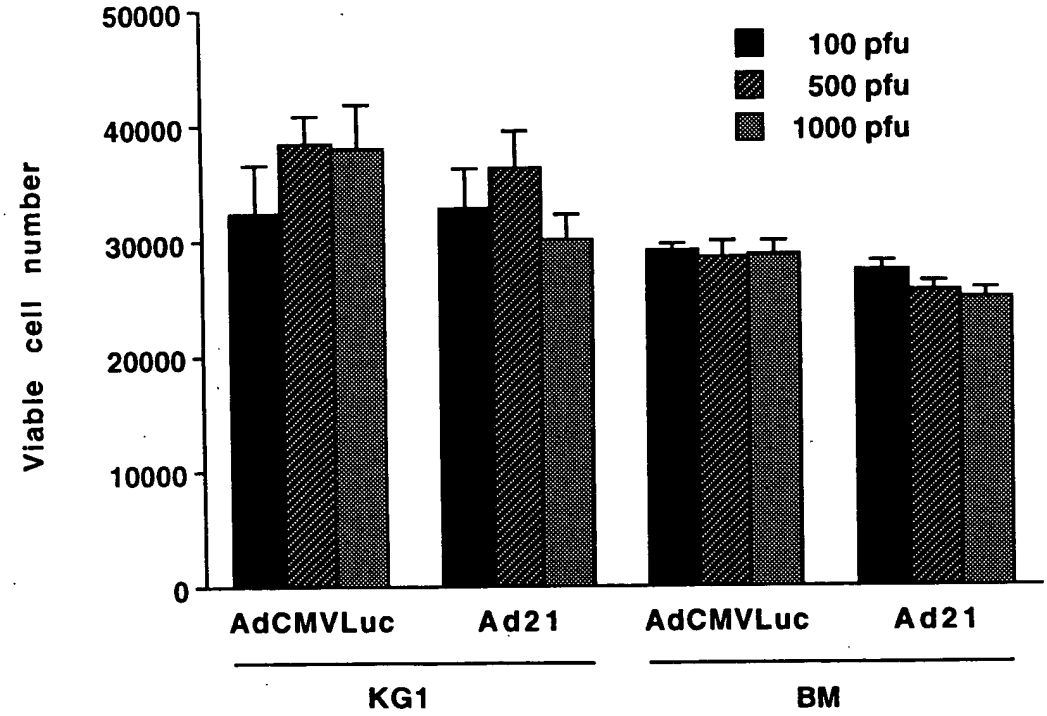


FIG. 6. Effect of various doses of anti-erbB-2 sFv-encoding adenovirus on human bone marrow cells. Human bone marrow cells were infected with the anti-erbB-2 sFv-encoding adenovirus (Ad21) at various moi values and analyzed for viability 96 hr post-transduction. Target cells included the KG-1 cell line as well as fresh human bone marrow cells. Experiments were performed five times with results reported as mean ± SEM.

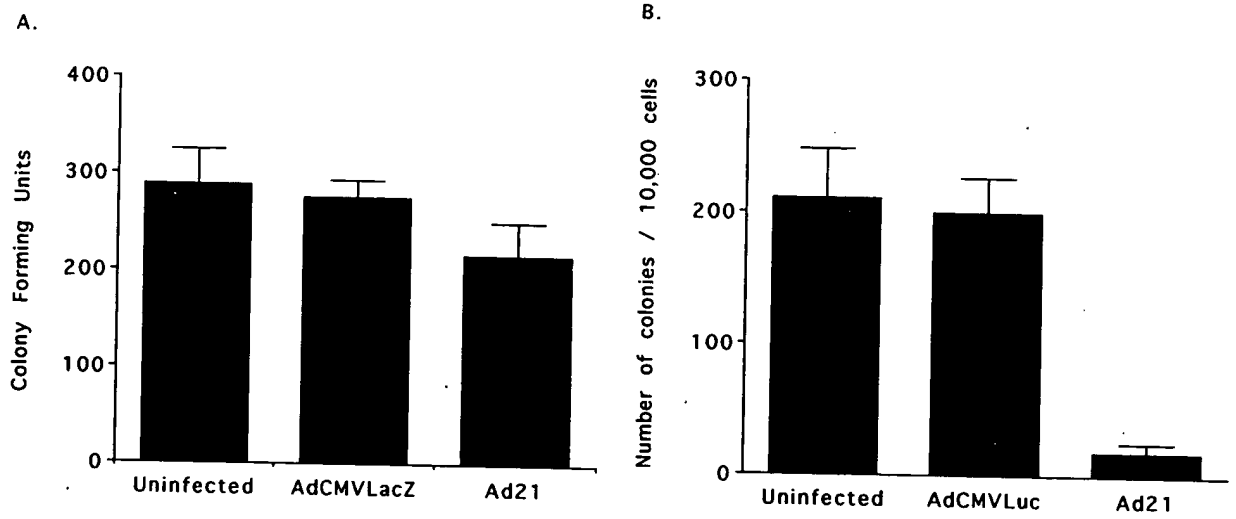


FIG. 7. Effect of anti-erbB-2 sFv-encoding adenovirus on bone marrow stem cell viability and clonogenicity of human prostate carcinoma cells. Human bone marrow cells and the human prostate carcinoma cell line DU 145 were infected with either a reporter gene encoding adenovirus (AdCMVLacZ or AdCMVLuc) or the anti-erbB-2 sFv-encoding adenovirus (Ad21) at an moi of 100 pfu/cell. A. Bone marrow cells were plated on methylcellulose agar-leukocyte-conditioned medium and assayed for CFU-GEMM colony formation at day 25. B. Prostate carcinoma cells were plated on soft agar and assayed for colony formation at day 21. Untransduced cells served as an additional control. Experiments were performed three times with results reported as mean ± SEM.

selective gene delivery to human prostate carcinoma cells without appreciable ectopic transduction of normal marrow cells was observed. Both candidate vector systems achieved nearly quantitative transduction of human prostate carcinoma cells whereas no detectable transduction of marrow cells was noted.

To this end, it has been reported that adenovirus is relatively ineffective in infecting lymphocyte and myelogenous cells (Horvath and Weber, 1988; Silver and Anderson, 1988; Haddada *et al.*, 1993; Huang *et al.*, 1995). In addition, a number of reports have noted the inability of cells of the

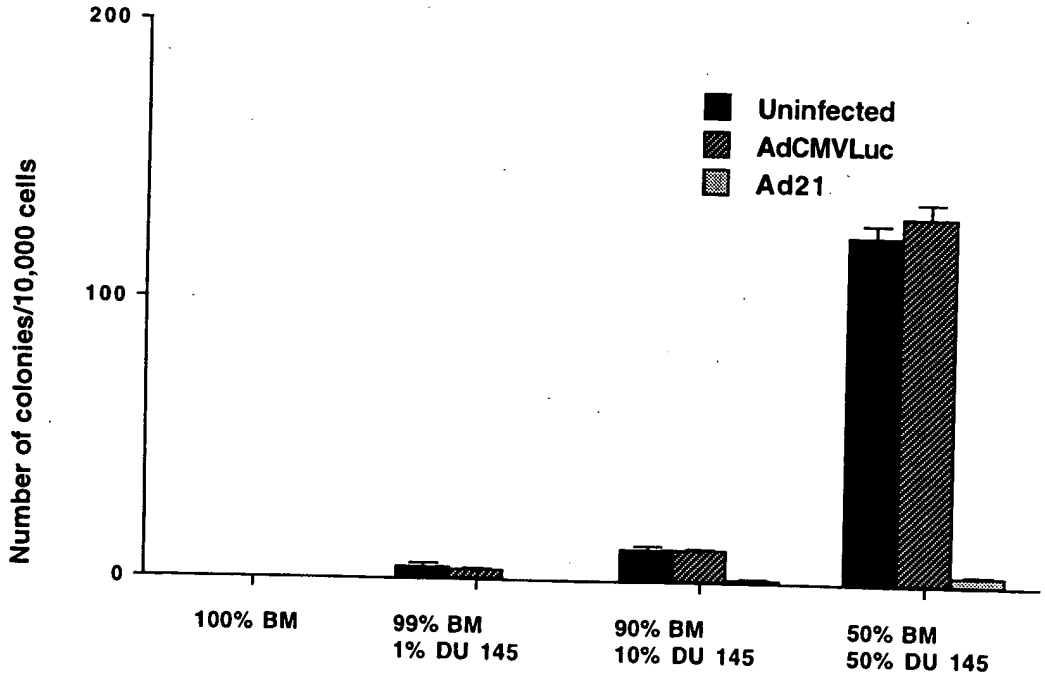


FIG. 8. Elimination of human prostate carcinoma cells from bone marrow. The human prostate carcinoma cell line DU 145 was mixed with fresh, irradiated human bone marrow cells at various ratios. Cell mixtures were then treated with either a reporter gene encoding adenovirus (AdCMVLuc) or an adenovirus encoding the anti-erbB-2 sFv (Ad21). Cells were plated in soft agar and assayed for colony formation at day 21. Untransduced cells served as an additional control. Adenoviral infections were carried out at an moi of 100 pfu per cell. Experiments were performed three times each with results reported as mean ± SEM.

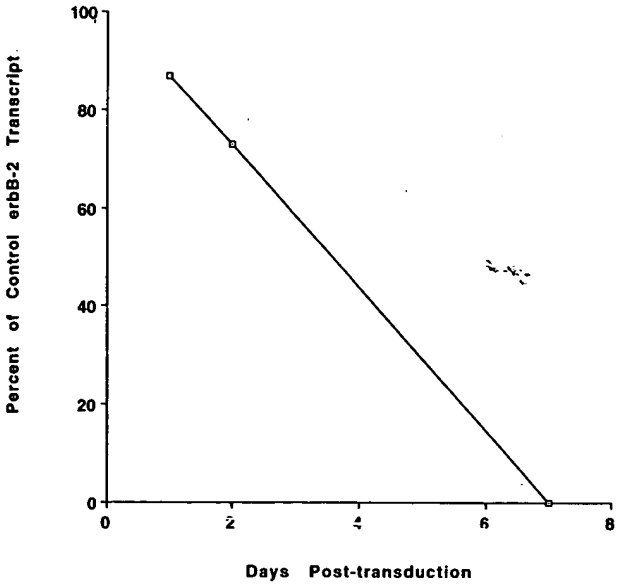


FIG. 9. Reduction of erbB-2 mRNA achieved in DU 145 cells treated with anti-erbB-2 sFv-encoding adenovirus. Human bone marrow spiked with 1% DU 145 cells was treated with the anti-erbB-2 sFv-encoding adenovirus Ad21. Target cells were then analyzed for total erbB-2 mRNA content at 1, 2, and 7 days post-transduction by QC-RT-PCR. Untransduced cells served as a control.

myelomonocytic lineage to sustain adenoviral infection over time (Faucon *et al.*, 1982; Chu *et al.*, 1992). Although some reports employ adenoviral vectors for transduction of monocytes and bone marrow cells (Mitani *et al.*, 1994), viral moi values employed were relatively high and overall transduction efficiencies were appreciably lower than those achieved for epithelial target cells. AdpL complexes are also relatively inefficient in accomplishing gene transfer to myelogenous cell targets (Kim *et al.*, in press), although this limitation may be partially circumvented by incorporation of specific targeting moieties into the AdpL complex (Curiel *et al.*, 1994). Nonetheless, it appears that these two candidate vectors possess a natural tropism profile whereby it can be predicted that they will not transduce bone marrow cells at a high efficiency. Therefore, this report demonstrates relative restriction of gene targeting to carcinoma cells while sparing marrow elements on the basis of transductional specificity of the vector system employed for purging.

The second necessary component of this purging strategy is based upon genetic induction of toxicity specifically in tumor targets. For this effect, an approach was employed that is based upon intracellular single-chain (sFv) antibody-mediated knockout of a specific gene product. To this end, we have developed a novel method to achieve targeted tumor toxicity based upon expression of an intracellular single-chain antibody (sFv) directed against the erbB-2 oncoprotein. This strategy would provide an additional means to spare normal marrow elements by

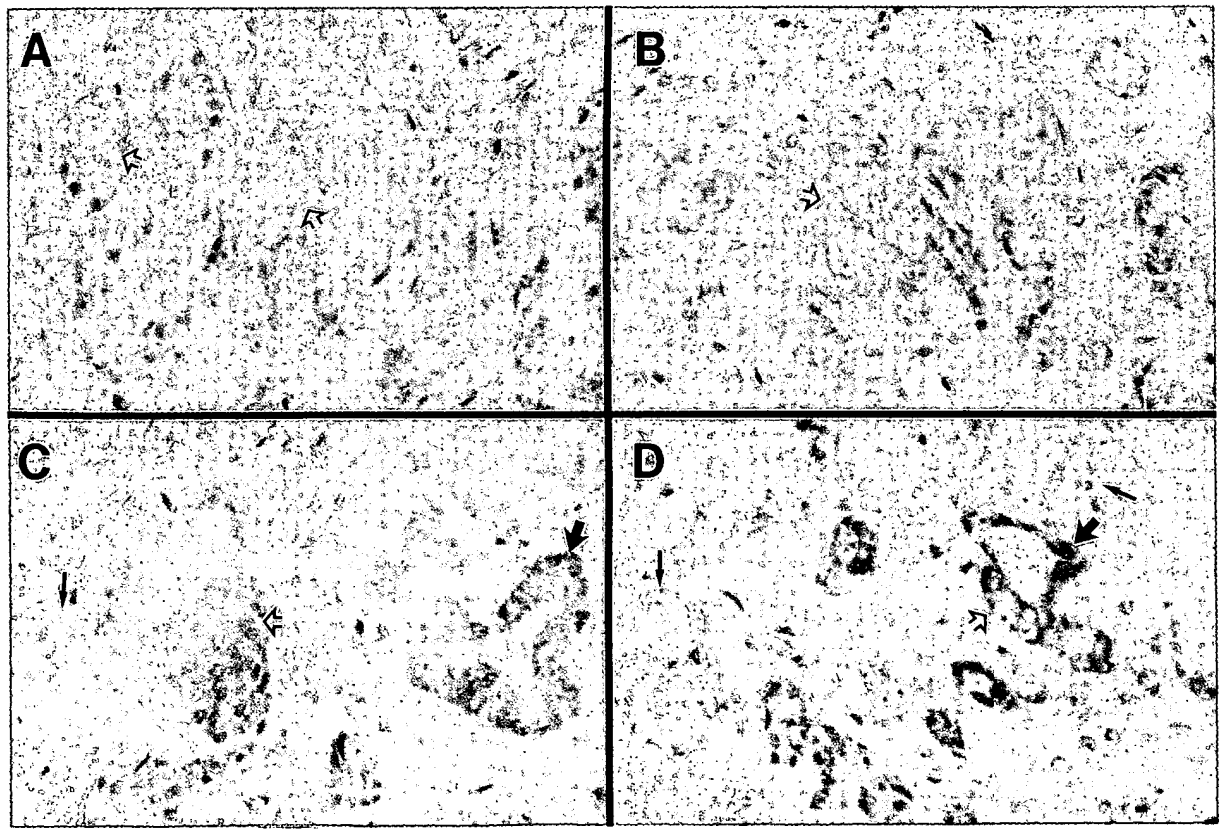


FIG. 10. Immunostaining for p185erbB-2 of primary prostate carcinoma. Sections of prostate carcinoma tissue resected from a patient were immunostained with a monoclonal antibody specific for human erbB-2. Thick arrows indicate strong staining, thin arrows indicate weak staining, open arrows indicate primarily membrane staining, and solid arrows indicate primarily cytoplasmic staining. A, B, C, and D represent different fields of the same biopsy.

assuring that vector-mediated nontumor transduction would not be associated with any cytotoxicity. Previous studies have demonstrated that this gene therapy approach induced apoptosis exclusively in erbB-2 overexpressing tumor cells (Deshane *et al.*, 1996; Wright *et al.*, in press). Other reports have also shown that single-chain antibody constructs specific for the erbB-2 gene product are an effective means to induce selective tumor cell toxicity in erbB-2-positive tumor cells derived from the ovary, breast, and lung (Deshane *et al.*, 1994; Grim *et al.*, 1996). In this study, expression of the anti-erbB-2 sFv induced a four-log reduction in viability of erbB-2-positive prostate tumor cells. The data here corroborate previous findings in which AdpL-mediated delivery of the anti-erbB-2 sFv resulted in selective eradication of the erbB-2-positive human prostate carcinoma cell lines DU 145 and LNCaP whereas the erbB-2-negative cell line PC-3 was spared. Although erbB-2 has not been demonstrated to play a key role in transformation in the context of carcinoma of the prostate, its overexpression nonetheless appears to sensitize tumor cells to sFv-mediated cytotoxicity.

An important aspect of the intracellular anti-oncogene knockout strategy is that it is highly specific. Thus, it has been shown for a large number of human tissues that lack erbB-2 overexpression that expression of the intracellular sFv is not associated with any toxicity. Moreover, erbB-2 expression has not been noted in the context of normal human bone marrow cells (Evinger-Hodges *et al.*, 1987; Di Fiore *et al.*, 1990). Therefore, it can be hypothesized that the anti-erbB-2 sFv, if expressed in these cells, would not be deleterious. To this end, ectopic transduction and killing of bone marrow progenitor cells would be counterproductive for ABMT therapy in the context of delivered genes that operate in a nonspecific manner. Here a genetic construct has been delivered whereby there was no observable bone marrow toxicity in total cell number nor progenitor cell viability, despite extremely effective tumor cell eradication. Thus, this post-transductional targeting schema offers an additional margin of selectivity within this purging strategy.

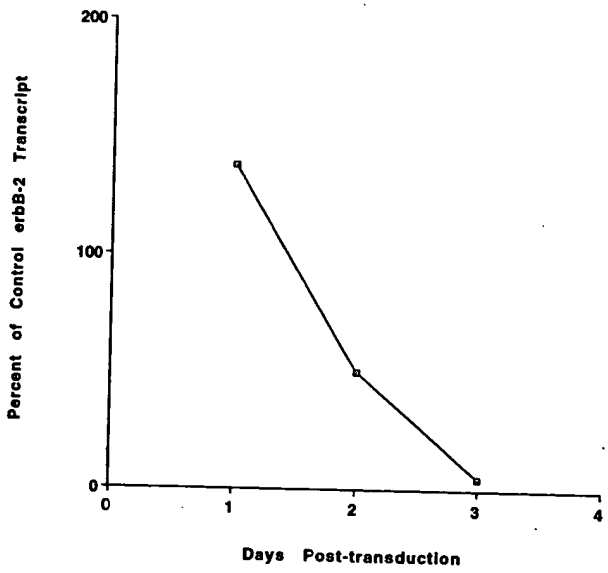


FIG. 11. Reduction of erbB-2 mRNA achieved in primary prostate carcinoma cells treated with anti-erbB-2 sFv-encoding adenovirus. Human bone marrow contaminated with erbB-2-positive refractory primary prostate carcinoma cells was treated with the anti-erbB-2 sFv encoding adenovirus, Ad21 at an moi of 100 pfu/cell. Target cells were analyzed at 1, 2, and 3 days post-treatment with Ad21 by QC-RT-PCR. Untransduced cells served as a control.

To evaluate better the efficacy of this novel purging strategy, a relatively rapid methodology to quantitate the degree of tumor target elimination was also developed. In this regard, QC-RT-PCR is increasingly being used both for detection of occult micrometastases in bone marrow harvests of ABMT candidates, and as a prognostic indicator of the utility of ABMT purge studies (Wood *et al.*, 1994; Stockschläder *et al.*, 1995). Using QC-RT-PCR, essential elimination of DU 145 cells from bone marrow mixing experiments was achieved. Although this data could be a simple down-modulation of erbB-2 mRNA levels, it closely agrees with the previous clonogenic assay results and suggests that specific tumor cell killing has occurred. In addition, a substantial reduction of erbB-2 mRNA levels was seen using QC-RT-PCR analysis on a bone marrow aspirate contaminated with refractory erbB-2 positive primary prostate carcinoma cells. Although residual erbB-2 mRNA could be detected in this preliminary study, careful optimization of assay conditions should allow the genetic purge strategy presented here to have potential for clinical efficacy.

This approach to marrow purge exploits two distinct gene therapy techniques to achieve selective elimination of tumors cells. By combining both a transductional and post-transductional approach, selective elimination of tumor targets was achieved. In this regard, a wide variety of methods have been developed to achieve elimination of tumor cells from marrow in the context of ABMT. In some of these instances, dramatic reduction of tumor cell contamination may be achieved, approaching a 4- to 5-order of magnitude elimination. In considering the present report in this context, it must be noted that many of the approaches employ panels of multiple antitumor antibodies and/or multistep purification schemas. Here, we report a novel conceptual approach to achieve this same end that may offer certain advantages compared to these earlier studies with respect to genetic specificity. In this regard, the efficacy of intracellular antibodies may not be as subservient to target antigen level, as conventional antibody delivered toxins (Wawrzynczak, 1992). In addition, it may be possible to target alternate markers in the event that disease is refractory to an sFv targeted to a primary tumor antigen. After further optimization, direct comparison to these earlier techniques will be feasible. Other investigators have also begun to explore viral-mediated transduction of tumor targets. Clarke *et al.* have recently reported that adenovirus-mediated delivery of the *bcl*-x_s gene eradicates a variety of epithelial tumors (Clarke *et al.*, 1995). Further, *bcl*-x_s's transduced marrow could fully reconstitute severe combined immunodeficiency disease (SCID) mice, demonstrating that the adenoviral vector has no untoward effect on any of the hematopoietic lineage. This study corroborates our concept that a genetic approach to marrow purge is possible. The present work extends this concept by the description of a second selection step. This bone marrow purge strategy, which utilizes both transductional and genetic specificity, provides an efficient means to remove erbB-2-positive

prostate tumor cells from bone marrow cells used for autologous bone marrow transplantation and provides a model for future *ex vivo* genetic purge applications.

ACKNOWLEDGMENTS

We wish to acknowledge the expert editorial assistance of Christi Stewart. In addition, we are indebted to Tracy James for assistance with FACS analysis, as well as Tracy Hammonds and Mary Patton of the Hematopoietic Stem Cell Processing Facility for provision of human bone marrow cells. We also thank Maryland Rosenfeld for her helpful discussions and critical reading of the manuscript.

This work was supported by the following grants: National Institutes of Health 1RO1CA725332-01 and U.S. Army DAMD 17-94-J-4398.

REFERENCES

BOCIEK, R.G., STEWART, D.A., and ARMITAGE, J.O. (1995). Bone marrow transplantation—current concepts. J. Invest. Med. **43,** 127–135.

BRODY, S.L., JAFFE, H.A., HAN, S.K., WERSTO, R.P., and CRYSTAL, R.G. (1994). Direct *in vivo* gene transfer and expression in malignant cells using adenovirus vectors. Hum. Gene Ther. **5,** 437–447.

CHU, Y., SPERBER, K., MAYER, L., and HUS, M-T. (1992). Persistent infection of human adenovirus type 5 in human monocyte cell lines. Virology **188,** 793–800.

CLARKE, M.F., APEL, I.J., BENEDICT, M.A., EIPERS, P.G., V., SUMANTRAN, V., GONZÁLEZ-GARCÍA, DOEDENS, M., FUKUNAGA, N., DAVIDSON, B., DICK, J.E., MINN, A.J., BOISE, L.H., THOMPSON, C.B., WICHA, M., and NUÑEZ, G. (1995). A recombinant bcl-x_s adenovirus selectively induces apoptosis in cancer cells but not in normal bone marrow cells. Proc. Natl. Acad. Sci. USA **92,** 11024–11028.

COLAK, A., GOODMAN, J.C., CHEN, S-H, WOO, S., GROSSMAN, R.G., and SHINE, H.D. (1995). Adenovirus-mediated gene therapy in an experimental model of breast cancer metastatic to the brain. Hum. Gene Ther. **6,** 1317–1322.

CURIEL, D.T., WAGNER, E., COTTEN, M., BIRNSTEIL, M.L., AGARWAL, S., LI, C-M, LOECHEL, S., and HU, P-C. (1992). High efficiency in vitro gene transfer mediated by an adenovirus coupled to DNA-polylysine complexes. Hum. Gene Ther. **3,** 147–154.

CURIEL, T.J., COOK, D.R., BOGEDAIN, C., JILG, W., HARRISON, G.S., COTTEN, D.T., CURIEL, D.T., and WAGNER, E. (1994). Efficient foreign gene expression in Epstein-Barr virus-transformed human B-cells. Virology **198,** 577–585.

DESHANE, J., LOECHEL, F., CONRY, R.M., SIEGAL, G.P., KING, C.R., and CURIEL, D.T. (1994). Intracellular single-chain antibody directed against erbB2 down-regulates cell surface erbB2 and exhibits a selective anti-proliferative effect in erbB2 overexpressing cell lines. Gene Ther. **1,** 332–337.

DESHANE, J., CABRERA, G., GRIM, J.E., SIEGAL, G.P., PIKE, J., ALVAREZ, R.D., and CURIEL, D.T. (1995a). Targeted eradication of ovarian cancer mediated by intracellular expression of anti-erbB-2 single-chain antibody. Gynecolog. Oncol. **59,** 8–14.

DESHANE, J., SIEGAL, G.P., ALVAREZ, R.D., WANG, M., FENG, M., CABRERA, G., LIU, T., KAY, M., and CURIEL, D.T. (1995b). Targeted tumor killing via an intracellular antibody against erbB-2. J. Clin. Invest. **96,** 2980–2989.

DESHANE, J., GRIM, J., LOECHEL, S., SIEGAL, G.P., ALVAREZ, R.D., KING, C.R., and CURIEL, D.T. (1996). Intracellular antibody directed against erbB-2 mediates tumor cell eradication by inducing apoptosis. Cancer Gene Ther. **3,** 89–98.

DI FIORE, P.P., SEGATTO, O., TAYLOR, W.G., AARONSON, S.A., and PIERCE, J.H. (1990). EGF receptor and erbB-2 tyrosine kinase domains confer cell specificity for mitogenic signaling. Science **248,** 79–82.

EVINGER-HODGES, M.J., BLICK, M., BRESSER, J., and DICKE, K.A. (1987). Comparison of oncogene expression in human normal bone marrow and leukemia. Ann. N.Y. Acad. Sci. **511,** 284–291.

FAUCON, N., OGIER, G., and CHARDONNET, Y. (1982). Changes in human adenovirus 5 propagated in Burkitt's Lymphoma cells. J. Natl. Cancer Inst. **69,** 1215–1220.

FENG, M., CABRERA, G., DESHANE, J., SCANLON, K.J., and CURIEL, D.T. (1995). Neoplastic reversion accomplished by high efficiency adenoviral-mediated delivery of an anti-*ras* ribozyme. Cancer Res **55,** 2024–2028.

GEE, A. (1995). Purging of peripheral blood stem cell grafts. Stem Cells **13**(Suppl. 3), 52–62.

GRIBBEN, J.G., and NADLER, L.M. (1993). Bone marrow purging for autologous bone marrow transplantation. Leuk. Lymphoma **11**(Suppl. 2), 141–148.

GRIM, J., DESHANE, J., FENG, M., LIEBER, A., KAY, M., and CURIEL, D.T. (1996). ErbB-2 knockout employing an intracellular single chain antibody (sFv) accomplishes specific toxicity in erbB-2 expressing lung cancer cells. Am. J. Respir. Cell. Mol. Biol. **15,** 348–354.

GROSS, H.-J., VERWER, B., HOUCK, D., HOFFMAN, R.A., and RECKENTENWALD, D. (1995). Model study detecting breast cancer cells in peripheral blood mononuclear cells at frequencies as low as 10^{-7}. Proc. Natl. Acad. Sci. USA **92,** 537–541.

HADDADA, H., LOPEZ, M., MARTINACHE, C., RAGOT, T., ABINA, M.A., and PERRICAUDET, M. (1993). Efficient adenovirus-mediated gene transfer into human blood monocyte-derived macrophages. Biochem. Biophys. Res. Commun. **195,** 1174–1183.

HOCKETT, JR., R.D., JANOWSKI, K.M., and BUCY, R.P. (1995). Simultaneous quantitation of multiple cytokine mRNAs by RT-PCR utilizing plate based EIA methodology. Immunol. Methods **187,** 273–285.

HORVATH, J., and WEBER, J.M. (1988). Nonpermissivity of human peripheral blood lymphocytes to adenovirus type 2 infection. J. Virol. **62,** 341–345.

HUANG, S., ENDO, R.I., and NEMEROW, G.R. (1995). Upregulation of integrins avb3 and avb5 on human monocytes and T lymphocytes facilitates adenovirus-mediated gene delivery J. Virol. **69,** 2257–2263.

JUNEJA, H.S., HARVEY, W.H., BRASHER, W.K., and THOMPSON, E.B. (1995). Successful *in vitro* purging of leukemic blasts from marrow by cortavizol, a pyrazolosteroid: A preclinical study for autologous transplantation in acute lymphoblastic leukemia and non-Hodgkin's lymphoma. Leukemia **9,** 1771–1778.

KIES, M.S., GORDON, L.I., ROSEN, S.T., KUCUK, O., and VRIESENDORP, H.M. (1988). Autologous bone marrow transplantation in breast cancer: separation of clonogenic tumor cell colonies by gradient fractionation. Exp. Hematol. **16,** 190–194.

KIM, M., ACAVITTI, M.A., SALEH, M.N., ROSENFELD, M.E., JOHANNING, F.-W., CURIEL, T.J., and CURIEL, D.T. (1996). Adenoviral vector mediated delivery of the herpes simplex virus thymidine kinase gene sensitizes EBV-transformed B cell lines to ganciclovir. Tumor Targeting (in press).

MEYER, B.F., STONER, M.L., RAPHAEL, C.L., DAVIS, R.E., and HERMANN, R.P. (1991). Strepavidin-biotin immunotoxins: a new approach to purging bone marrow. Exp. Hematol. **19,** 710–713.

MITANI, K., GRAHAM, F.L., and CASKEY, C.T. (1994). Transduction of human bone marrow by adenoviral vector. Hum. Gene Ther. **5,** 941–948.

MORIYAMA, Y., GOTO, T., HASHIMOTO, S., T. FURUKAWA, T.,

KISHI, K., TAKAHASHI, M., and SHIBATA, A. (1992). A simple elimination of clonogenic tumor cells from human bone marrow in vitro by heat: Its application to autologous bone marrow transplantation for B-cell lymphoma. Ann. Hematol. **64,** 266–269.

MYKLEBUST, A.T., GODAL, A., JUELL, S., PHARO, A., and FODSTAD, Ø. (1994). Comparison of two antibody-based methods for elimination of breast cancer cells from human bone marrow. Cancer Res. **54,** 209–214.

O'BRIANT, K.C., SHPALL, E.J., HOUSTON, L.L., PETERS, W.P., and BAST, R.C. (1991). Elimination of clonogenic breast cancer cells from human bone marrow: A comparison of immunotoxin treatment with chemoimmunoseparation using 4-hydroperoxy-cyclophosphamide, monoclonal antibodies, and magnetic microspheres. Cancer **68,** 1272–1278.

PETERS, W.B., ROSS, M., VREDENBURGH, J.J., MEISENBERG, B., MARKS, L.B., WINER, E., KURTZBERG, J., BAST, R.C., JONES, JR., R. SHPALL, E., WU, K., ROSNER, G., GILBERT, C., MATTHIAS, B., CONIGLIO, D., PETROS, W., HENDERSON, I.C., NORTON, L., WEISS, R.B., BUDMAN, D., and HURD, D. (1993). High-dose chemotherapy and autologous bone marrow support as consolidation after standard-dose adjuvant therapy for high-risk primary breast cancer. J. Clin. Oncol. **11,** 1132–1143.

ROSENFELD, M.R., FENG, M., MICHAEL, S.I., SIEGAL, G.P., ALVAREZ, R.D., and CURIEL, D.T. (1995). Adenoviral mediated delivery of the HSV thymidine kinase gene selectively sensitizes human ovarian carcinoma cells to ganciclovir. Clin. Cancer Res. **1,** 1571–1580.

SILVER, L., and ANDERSON, C.W. (1988). Interaction of human adenovirus serotype 2 with human lymphoid cells. Virology **165,** 377–387.

SIMPSON, S.J., VACHULA, M., KENNEDY, M.J., KAIZER, H., COON, J.S., GHALIE, R., WILLIAMS, S., and VAN EPPS, D. (1995). Detection of tumor cells in the bone marrow, peripheral blood, and apheresis products of breast cancer patients using flow cytometry. Exp. Hematol. **23,** 1062–1068.

STOCKSCHLÄDER, M., HEGEWISCH-BECKER, S., KRUGER, W., TOM DIECK, A., MROSS, K., HOFFKNECHT, M., BERGER, C., KOHLSCHÜTTER, B., MARTIN, H., PETERS, S., KABISCH, H., KUSE, R., WEH, H., and ZANDER, A. (1995). Bone marrow transplantation for Philadelphia-chromosome-positive acute lymphoblastic leukemia. Bone Marrow Transplant. **16,** 663–667.

WARWRZYNCZAK, E.J. (1992). Rational design of immunotoxins: Current progress and future prospects. Anti-Cancer Drug Design **7,** 427–441.

WOOD, D.P., JR., BANKS, E.R., HUMPHREYS, S., MCROBERTS, J.W., and RANGNEKAR, V.M. (1994). Identification of bone marrow micrometastases in patients with prostate cancer. Cancer **74,** 2533–2540.

Address reprint requests to:
Dr. David T. Curiel
University of Alabama at Birmingham
Gene Therapy Program
1824 6th Avenue South, WTI 620
Birmingham, AL 35294

Received for publication May 14, 1996; accepted after revision November 4, 1996.

STIC-ILL RC 261. A4313

From: Hunt, Jennifer
Sent: Tuesday, January 22, 2002 5:54 PM
To: STIC-ILL
Subject: References for 09/602,800

Please send me the following references ASAP:

Hum. Gene Ther. (1997), 8(2), 157-170

CLINICAL CANCER RESEARCH, (1999 Aug) 5 (8) 2171-7

CANCER RESEARCH, (1999 Oct 1) 59 (19) 4761-4

SEMINARS IN ONCOLOGY, (2000 Dec) 27 (6 Suppl 11) 53-63; discussion 92-100

SEMINARS IN ONCOLOGY, (2001 Aug) 28 (4 Suppl 15) 3-7

SEMINARS IN ONCOLOGY, (2001 Aug) 28 (4 Suppl 15) 71-6

Thanks,

Jennifer Hunt
Patent Examiner, Art Unit 1642
CM1-8D06 (mailbox 8E12)
(703)308-7548

Preclinical Mechanisms of Action of Docetaxel and Docetaxel Combinations in Prostate Cancer

Kenneth J. Pienta

Docetaxel, a semisynthetic taxane, has exhibited significant single-agent activity against prostatic tumors. In phase I/II studies, single-agent docetaxel and the combination of docetaxel plus estramustine were effective in inducing prostate-specific antigen reductions of ≥50% in men with androgen-independent prostate cancer (AIPC). The underlying reason for docetaxel's clinical activity against prostate cancer has been a focus of ongoing research. Docetaxel is believed to have a two-fold mechanism of antineoplastic activity: (1) inhibition of microtubular depolymerization, and (2) attenuation of the effects of bcl-2 and bcl-xL gene expression. Taxane-induced microtubule stabilization arrests cells in the G_2M phase of the cell cycle and induces bcl-2 phosphorylation, thereby promoting a cascade of events that ultimately leads to apoptotic cell death. In preclinical studies, docetaxel had a higher affinity for tubulin and was shown to be a more potent inducer of bcl-2 phosphorylation than paclitaxel. Laboratory evidence also supports the clinical evaluation of docetaxel-based combinations that include agents such as trastuzumab and/or estramustine. The pathways for docetaxel-induced apoptosis appear to differ in androgen-dependent and androgen-independent prostate cancer cells. Further elucidation of these differences will be instrumental in designing targeted regimens for the treatment of localized and advanced prostate cancer.
Semin Oncol 28 (suppl 15):3-7. ***Copyright © 2001 by W.B. Saunders Company.***

PROSTATE CANCER continues to be a major cause of morbidity among older men in the United States and is expected to result in 31,500 deaths in 2001.[1] Prostatic tumors generally respond to initial androgen ablation therapy; however, this treatment exerts preferential effects against androgen-dependent cells, while androgen-independent cells continue to thrive. These androgen-independent cells are responsible for the disease relapse associated with advanced prostate cancer. Chemotherapy is one of the main treatments of choice for hormone-refractory prostate cancer (HRPC) or androgen-independent prostate cancer (AIPC). Until recently, cytotoxic chemotherapy was viewed as a rather ineffective approach to prostate cancer management, but newer agents have demonstrated the potential for enhanced clinical activity in this setting.[2]

CLINICAL STUDIES OF DOCETAXEL IN PROSTATE CANCER PATIENTS

Docetaxel (Taxotere; Aventis Pharmaceuticals Inc, Bridgewater, NJ) has shown promise for the treatment of AIPC when used as monotherapy or in combination with other chemotherapeutic agents. The approved dosage range for breast cancer is 60 to 100 mg/m^2 administered as a 1-hour intravenous infusion every 3 weeks.[3] In a recent phase II study by Friedland et al,[4] 38% of patients receiving docetaxel, 75 mg/m^2 intravenously (IV) every 3 weeks, for AIPC achieved a prostate-specific antigen (PSA) level decrease of ≥50%, and more than 50% of patients experienced symptomatic improvement. Picus and Shultz[5] also evaluated docetaxel, 75 mg/m^2 IV, in 35 patients with AIPC[5]; 46% of patients experienced PSA level decreases of ≥50%, with seven patients achieving PSA reductions of >80%.[5]

Clinical studies have evaluated the combination of docetaxel and estramustine in patients with AIPC. A small-scale phase I study by Kreis and Budman[6] showed that 82% of patients with metastatic AIPC who received docetaxel (40 to 80 mg/m^2 IV) plus oral estramustine (14 mg/kg) daily achieved PSA reductions of ≥50%. In a phase I study by Petrylak et al,[7] docetaxel (40 to 80 mg/m^2 IV on day 2) plus a 5-day course of oral estramustine (280 mg three times daily on days 1 through 5) every 3 weeks produced PSA reductions of ≥50% in 70% of patients with minimally pretreated AIPC and 50% of those with heavily pretreated disease.

These promising clinical data for docetaxel and

From the Department of Internal Medicine, Division of Hematology/Oncology, University of Michigan, Ann Arbor, MI.
Supported by CaPCURE and SPORE grant P50 CA69568.
Address reprint requests to Kenneth J. Pienta, MD, Department of Internal Medicine, Division of Hematology/Oncology, University of Michigan, 1500 E Medical Center Dr, Rm 7303 Cancer Center, Ann Arbor, MI 48109-0946.
Copyright © 2001 by W.B. Saunders Company
0093-7754/01/2804-1502$35.00/0
doi:10.1053/sonc.2001.26892

docetaxel/estramustine in patients with AIPC lead us to question why docetaxel appears to be more effective than other chemotherapeutic agents with activity against this difficult-to-treat disease.

PRECLINICAL STUDIES OF DOCETAXEL

Docetaxel, a semisynthetic taxane, is believed to have a twofold mechanism of antineoplastic activity.[2,8] First, docetaxel inhibits microtubular depolymerization; second, it has been found to counter the effects of bcl-2 and bcl-xL gene expression.[9,10] Microtubule stabilization, the most widely accepted mechanism of action, involves the binding of docetaxel to β-tubulin, thus promoting polymerization.[11]

Under normal conditions, microtubules undergo polymerization in the presence of microtubule-associated proteins and guanosine triphosphate (GTP), which interacts with β-tubulin.[12] Taxanes, however, have been shown to bind preferentially to β-tubulin,[13] leading to microtubule assembly in the absence of GTP and other cofactor proteins.[14] Once bound by taxanes, microtubules cannot be disassembled, even at 4°C or in the presence of Ca^{2+} ions (two standard in vitro methods used for microtubule depolymerization).[13,15] This static polymerization disrupts the normal mitotic process and typically arrests cells in the G_2M phase of the cell cycle, ultimately leading to apoptosis.

Docetaxel has demonstrated a greater and more slowly reversible degree of polymerization than paclitaxel.[8] The equilibrium constant for tubulin binding also favors docetaxel,[12] suggesting that it has a somewhat higher affinity for tubulin than paclitaxel and is a more potent inducer of microtubule assembly. The dissociation constant for the binding of docetaxel to tubulin also is much greater than the LD_{50} in several prostate cancer cell lines (LNCaP and DU-145).[10] Therefore, it has been hypothesized that docetaxel concentrates within cells,[16,17] making it a more potent microtubule stabilizer intracellularly than extracellularly.

It is well established that docetaxel arrests cells in the G_2M phase of the cell cycle. Cells that undergo this cell cycle arrest also exhibit bcl-2 phosphorylation, an additional apoptotic marker. The bcl-2 gene is a member of a novel class of oncogenes that contribute to neoplastic progression by enhancing tumor cell survival through inhibition of apoptotic cell death.[18] We have previously shown that bcl-2 normally is phosphorylated as part of the G_2/M interface. We theorize that this temporary event provides for activation of the caspase cascade, thereby facilitating mitosis via the dissolution of lamins and other cell structure proteins. The taxanes, by inducing bcl-2 phosphorylation, force continued activation of the caspase cascade, leading to increased apoptosis (Fig 1). Bcl-2 dimerizes with bax, a proapoptotic protein, and subsequently inhibits its function.[19] It has been demonstrated that bcl-2 overexpression protects prostate cancer cells from apoptosis after androgen withdrawal; therefore, bcl-2 has become a target for antisense technology.[20] Several experimental and clinical studies suggest that enhanced bcl-2 expression confers both chemoresistance and androgen resistance and, in effect, may lead to androgen independence.[21,22] In 1995, Haldar et al[23] demonstrated that phosphorylation of bcl-2 at serine residues leads to loss of bcl-2 antiapoptotic function. This phenomenon is thought to occur through decreased bcl-2 binding to the proapoptotic bax protein.[24] Taxane-induced microtubule stabilization appears to induce bcl-2 phosphorylation in cancer cells, followed by an increase in free bax and apoptosis.[24] In prostate cancer cells, docetaxel is more potent than paclitaxel in inducing bcl-2 phosphorylation and apoptotic death.[9]

In a recent study by Krajewski et al,[25] the antiapoptotic protein bcl-xL was expressed in all clinical prostate cancers (64 of 64). Although docetaxel induces phosphorylation of bcl-xL in several cell lines, this does not occur in LNCaP and PC-3 prostate cancer cells.[26] Instead, docetaxel appears to decrease bcl-xL protein and messenger RNA expression.[10] Many have speculated that these effects are capable of sensitizing cells to other chemotherapeutic agents; however, this remains unclear.

PRECLINICAL RATIONALE FOR CURRENT AND FUTURE TAXOTERE COMBINATIONS IN PROSTATE CANCER

Burris[27] recently showed a true in vitro and in vivo synergistic interaction between docetaxel and trastuzumab (Herceptin; Genentech, Inc, South San Francisco, CA), a recombinant human anti-HER2 monoclonal antibody. HER2 status has shown utility for identifying those patients most likely to derive benefit from chemotherapy and for

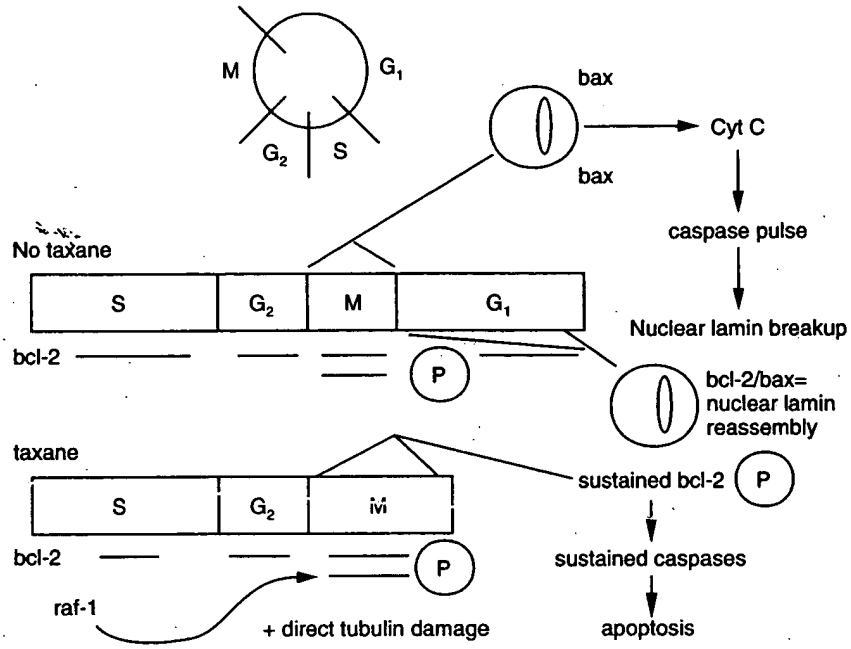


Fig 1. Taxane-induced apoptosis through the cell cycle.

monitoring the effectiveness of treatment. Estramustine-based combination chemotherapy has demonstrated substantial therapeutic potential in the management of prostate cancer.[28,29] In phase II studies, PSA reductions up to 60% were reported in patients receiving estramustine plus vinblastine and/or etoposide for AIPC.[28,29] Traditionally, estramustine phosphate was thought to exert

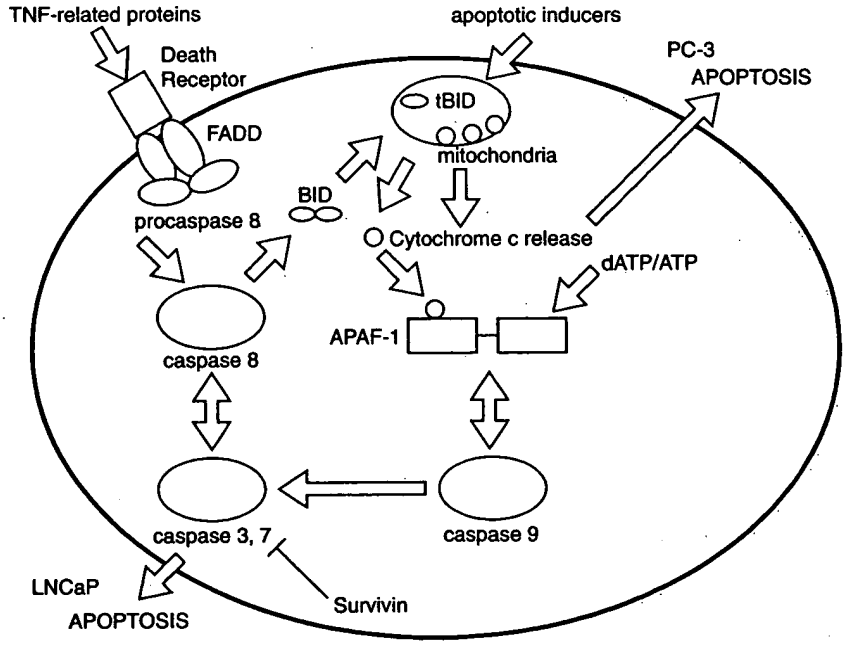


Fig 2. Docetaxel-induced apoptotic pathways in LNCaP and PC-3 cells.[33] BID, BH3-interacting domain death agonist; FADD, Fas-associated death domain protein; APAF-1, apoptosis protease-activating factor 1.

its antitumor activity by interfering with microtubule function. However, recent studies have found that estramustine metabolites bind to mutated androgen receptors in LNCaP cells and inhibit their function by blocking receptor phosphorylation.[30,31] A recent in vitro study by Williams et al[32] showed that the combination of docetaxel and estramustine exerts significant cytotoxic effects in PC-3 and MatLyLu prostatic cell lines.

Because prostatic tumors are composed of both androgen-dependent and androgen-independent cells, the therapeutic efficacy of a given biologic, hormonal, or cytotoxic agent may depend in part on the relative proportions of these cells. Recently, Muenchen et al[33] demonstrated that the pathways for docetaxel-induced apoptosis differ between androgen-responsive LNCaP cells and androgen-independent PC-3 cells. LNCaP cells appeared to cleave caspase-3 and -7, whereas PC-3 cells cleaved caspase-8 (Fig 2). Furthermore, survivin, an antiapoptotic marker, has been shown to be expressed constitutively in PC-3 cells but not in LNCaP cells, which instead express the apoptotic marker, clusterin.[33] The stark contrast between the apoptotic pathways within these two cell lines will aid researchers in identifying specific therapies with activity against localized versus advanced prostate cancer.

CONCLUSION

The future of docetaxel and docetaxel-based combinations as a component of prostate cancer management is promising. In recent years, clinical trials have demonstrated that docetaxel, 75 mg/m^2 IV every 3 weeks, is effective in inducing PSA responses $\geq$50% in patients with AIPC. Docetaxel-based combination chemotherapy appears to be more effective than single-agent docetaxel in this setting. Recent preclinical studies have improved our understanding of docetaxel's pronounced antineoplastic activity against prostatic tumors. The antitumor activity of docetaxel in patients with prostate cancer and other solid tumors has stimulated further research into the cytotoxic properties of this novel taxane.

REFERENCES

1. Greenlee RT, Hill-Harmon MB, Murray T, et al: Cancer statistics, 2001. CA Cancer J Clin 51:15-36, 2001

2. Oh WK, Kantoff PW: Management of hormone refractory prostate cancer: Current standards and future prospects. J Urol 160:1220-1229, 1998

3. Earhart RH: Docetaxel (Taxotere): Preclinical and general clinical information. Semin Oncol 26:8-13, 1999 (suppl 17)

4. Friedland D, Cohen J, Miller R Jr, et al: A phase II trial of docetaxel (Taxotere) in hormone-refractory prostate cancer: Correlation of antitumor effect to phosphorylation of Bcl-2. Semin Oncol 26:19-23, 1999 (suppl 17)

5. Picus J, Shultz M: Docetaxel (Taxotere) as monotherapy in the treatment of hormone-refractory prostate cancer: Preliminary results. Semin Oncol 29:14-18, 1999 (suppl 17)

6. Kreis W, Budman D: Daily oral estramustine and intermittent intravenous docetaxel (Taxotere) as chemotherapeutic treatment for metastatic, hormone-refractory prostate cancer. Semin Oncol 26:34-38, 1999 (suppl 17)

7. Petrylak DP, Macarthur RB, O'Connor J, et al: Phase I trial of docetaxel with estramustine in androgen-independent prostate cancer. J Clin Oncol 17:958-967, 1999

8. Ringel I, Horwitz SB: Studies with RP 56976 (Taxotere): A semisynthetic analogue of taxol. J Natl Cancer Inst 83:288-291, 1991

9. Haldar S, Basu A, Croce CM: Bcl2 is the guardian of microtubule integrity. Cancer Res 57:229-233, 1997

10. Stein CA: Mechanisms of action of taxanes in prostate cancer. Semin Oncol 26:3-7, 1999 (suppl 17)

11. Riou J, Petitgenet O, Combeau C, et al: Cellular uptake and efflux of docetaxel (Taxotere) and paclitaxel (Taxol) in P388 cell line. Proc Am Assoc Cancer Res 35:385, 1994 (abstr)

12. Diaz JF, Andreu JM: Assembly of purified GDP-tubulin into microtubules induced by taxol and taxotere: Reversibility, ligand stoichiometry, and competition. Biochemistry 32:2747-2755, 1993

13. Horwitz SB: Taxol (paclitaxel): Mechanisms of action. Ann Oncol 5:S3-S6, 1994 (suppl 6)

14. Manfredi JJ, Horwitz SB: Taxol: An antimitotic agent with a new mechanism of action. Pharmacol Ther 25:83-125, 1984

15. Manfredi JJ, Parness J, Horwitz SB: Taxol binds to cellular microtubules. J Cell Biol 94:688-696, 1982

16. Jordan MA, Toso RJ, Thrower D, et al: Mechanisms of mitotic block and inhibition of cell proliferation by Taxol at low concentrations. Proc Natl Acad Sci USA 90:9552-9556, 1993

17. Jordan MA, Wendell K, Gardiner S, et al: Mitotic block induced in HeLa cells by low concentrations of paclitaxel (Taxol) results in abnormal mitotic exit and apoptotic cell death. Cancer Res 56:816-825, 1996

18. Tsujimoto Y, Croce CM: Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. Proc Natl Acad Sci USA 83: 5214-5218, 1986

19. Sato N, Sadar MD, Bruchovsky N, et al: Androgenic induction of prostate-specific antigen gene is repressed by protein-protein interaction between the androgen receptor and AP-1/c-Jun in the human prostate cancer cell line LNCaP. J Biol Chem 272:17485-17494, 1997

20. Raffo AJ, Perlman H, Chen MW, et al: Overexpression of bcl-2 protects prostate cancer cells from apoptosis in vitro and confers resistance to androgen depletion in vivo. Cancer Res 55:4438-4445, 1995

21. McDonnell TJ, Troncoso P, Brisby SM, et al: Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. Cancer Res 52:6940-6944, 1992

22. Berchem GJ, Bosseler M, Sugars LY, et al: Androgens induce resistance to bcl-2-mediated apoptosis in LNCaP prostate cancer cells. Cancer Res 55:735-738, 1995

23. Haldar S, Jena N, Croce CM: Inactivation of Bcl-2 by phosphorylation. Proc Natl Acad Sci USA 92:4507-4511, 1995

24. Halder S, Chintapalli J, Croce CM: Taxol induces bcl-2 phosphorylation and death of prostate cancer cells. Cancer Res 56:1253-1255, 1996

25. Krajewski S, Krajewska M, Shabaik A, et al: Immunohistochemical analysis of in vivo patterns of Bcl-x expression. Cancer Res 54:5501-5507, 1994

26. Liu QY, Stein CA: Taxol and estramustine-induced modulation of human prostate cancer cell apoptosis via alteration in bcl-xL and bak expression. Clin Cancer Res 3:2039-2046, 1997

27. Burris HA: Docetaxel (Taxotere) in HER-2-positive patients and in combination with trastuzumab (Herceptin). Semin Oncol 27:19-23, 2000 (suppl 3)

28. Hudes GR, Greenberg R, Krigel RI, et al: Phase II study of estramustine and vinblastine, two microtubule inhibitors, in hormone-refractory prostate cancer. J Clin Oncol 10:1754-1761, 1992

29. Pienta KJ, Redman B, Hussain M, et al: Phase II evaluation of oral estramustine and oral etoposide in hormone-refractory adenocarcinoma of the prostate. J Clin Oncol 12: 2005-2012, 1994

30. Wang LG, Liu XM, Kreis W, et al: Androgen antagonistic effect of estramustine phosphate (EMP) metabolites on wild-type and mutated androgen receptor. Biochem Pharmacol 55:1427-1433, 1998

31. Wang LG, Liu XM, Kreis W, et al: Phosphorylation/dephosphorylation of androgen receptor as a determinant of androgen agonistic or antagonistic activity. Biochem Biophys Res Commun 259:21-28, 1999

32. Williams JF, Muenchen HJ, Kamradt JM, et al: Treatment of androgen-independent prostate cancer using antimicrotubule agents docetaxel and estramustine in combination: An experimental study. Prostate 44:275-278, 2000

33. Muenchen HJ, Poncza PJ, Pienta KJ: Different docetaxel-induced apoptotic pathways are present in prostate cancer cell lines LNCaP and PC-3. Urology 57:366-370, 2001